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**Citation for published version:**

de Castro, M, Martinez-Rubio, R, Luis Acebes, J, Encina, A, Fry, S & Garcia-Angulo, P 2017, 'Phenolic metabolism and molecular mass distribution of polysaccharides in cellulose-deficient maize cells' *Journal of integrative plant biology*, vol. 59, no. 7, pp. 475-495. DOI: 10.1111/jipb.12549

**Digital Object Identifier (DOI):**

[10.1111/jipb.12549](https://doi.org/10.1111/jipb.12549)

**Link:**

[Link to publication record in Edinburgh Research Explorer](#)

**Document Version:**

Peer reviewed version

**Published In:**

*Journal of integrative plant biology*

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**Phenolic metabolism and molecular mass distribution of polysaccharides in cellulose-deficient maize cells**

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**Keywords:** Arabinoxylan; cell wall cross-linking; 2,6-dichlorobenzonitrile; hemicellulose biosynthesis; ferulic acid; xyloglucan

Cell wall reinforcement in cellulose-deficient maize cells

20

21 **ABSTRACT**

22 As a consequence of the habituation to low levels of dichlobenil (DCB), cultured maize  
23 cells presented an altered hemicellulose cell fate with a lower proportion of strongly wall-  
24 bound hemicelluloses and an increase in soluble extracellular polymers released into the  
25 culture medium. The aim of this study was to investigate the relative molecular mass  
26 distributions of polysaccharides as well as phenolic metabolism in cells habituated to low  
27 levels of DCB (1.5 uM). Generally, cell wall bound hemicelluloses and sloughed polymers  
28 from habituated cells were more homogeneously sized and had a lower weight-average  
29 relative molecular mass. In addition, polysaccharides underwent massive cross-linking after  
30 being secreted into the cell wall, but this cross-linking was less pronounced in habituated  
31 cells than in non-habituated ones. However, when relativized, ferulic acid and *p*-coumaric  
32 acid contents were higher in this habituated cell line. Feasibly, cells habituated to low levels  
33 of DCB synthesized molecules with a lower weight-average relative molecular mass,  
34 although cross-linked, as a part of their strategy to compensate for the lack of cellulose.

35

## INTRODUCTION

Heteroxylans are the main non-cellulosic polysaccharides in the Poales primary (type II) cell wall (Fincher 2009; Peña et al. 2016), playing a major structural role in tethering cellulose microfibrils; hence, they are involved in cell expansion and plant growth. Heteroxylans show greater structural diversity and complexity than other types of cell wall components, since a wide range of different side-chain substitutions are found connected to the backbone (Faik 2010). These non-cellulosic polysaccharides are predicted to be synthesized in Golgi apparatus by several glycosyltransferases whose genes have been recently identified in *A. thaliana* by using genomic approaches (Rennie and Sheller 2014 and refs. therein). Specifically in the case of maize, sequences have also been reported for some arabidopsis orthologues such as *IRX9*, *IRX10* and *IRX10-L* (Bosch et al. 2011), and recently maize genes orthologous to AtIRX10, AtIRX9 and AtIRX14 has been shown to be highly expressed in the pericarp (Chateigner-Boutin et al. 2016).

One of the unique structural features of arabinoxylan in Poales is that it contains hydroxycinnamate residues, such as ferulic acid or *p*-coumaric acid, esterified on the C-5 position of arabinose residues (Bacic et al. 1988; Wende and Fry 1997). The attachment of ferulic acid residues occurs within the protoplasm, before secretion into the cell wall (Fry et al. 2000). Ferulic acid may undergo oxidative coupling when exposed to hydrogen peroxide plus peroxidase (Geissmann and Neukom 1971). Dimerisation of feruloyl polysaccharides may occur in the protoplasm before polysaccharides are secreted into the cell wall, in the cell wall just after secretion, or later in the cell wall after binding to the wall (Fry et al. 2000; Mastrangelo et al. 2009). Larger coupling products, such as trimers or oligomers, are also formed and it has been described to predominate over diferulates (Fry et al. 2000). Phenolic residues are also attached to polysaccharides as ether-like (alkali-stable) bonds (Burr and Fry 2009). Since they are thought to be involved in the heteroxylan cross-linking, they are essential for important biological processes such as wall assembly and restriction of cell expansion (Fry 2004; Parker et al. 2005; Oliveira et al. 2015).

One constraint industries based on the use of grass-derived products is cell wall digestibility, which is highly related to heteroxylan content, structure and interaction with the components of the remaining cell wall forming networks (Oliveira et al. 2015). Despite notable advances in our knowledge about heteroxylan synthesis and assembly in recent years, some aspects still remain unclear. Elucidation of its metabolism is particularly

challenging, and also has important economic implications. In line with this, the use of cell cultures habituated to cellulose biosynthesis inhibitors (for a review see Acebes et al. 2010) such as 2,6-dichlorobenzonitrile (dichlobenil, DCB) has been demonstrated to be a valuable tool to gain insight into the mechanism responsible for the metabolic and structural plasticity exhibited by plant cell walls.

DCB habituation is a dynamic process based on the capacity of cultured cells to implement coping strategies in order to survive with a cellulose-deficient wall. In the case of maize suspension-cultured cells, a quantitatively and qualitatively re-structured and modified arabinoxylan network is involved in many of these strategies (Mélida et al. 2009). In addition, the features and processes involved of the coping strategies exhibited in these cultured cells depend on DCB habituation level, which is highly related to the magnitude of the reduction in cellulose content. Likewise, in high DCB levels (12  $\mu\text{M}$ ) a 75 % of reduction is observed (Mélida et al. 2009) whereas in low DCB levels (1.5  $\mu\text{M}$ ) such reduction is more moderate, being of 33 % (de Castro et al. 2014) when compared to non-habituated maize cells (Shn). Hydroxycinnamates seems to play a crucial role in strengthening cellulose-deficient cell walls in maize cells habituated to high DCB levels, as they are involved in polysaccharide cross-linking (Mélida et al. 2010a, 2010b, 2011).

Cell suspension cultures present several advantages in *in vivo* metabolic studies performed using isotopes, such as homogeneity of cell wall material, uniform culture medium without nutrient gradients, access to  $\text{O}_2$  or exogenous precursors, and ease of sampling (Burr and Fry 2009). This kind of experimental approaches is particularly useful as the substrates are endogenous and therefore the processes observed are those that naturally take place (Thompson and Fry 1997; Kerr and Fry 2003, 2004; Lindsay and Fry 2008; Mélida et al. 2011). In a previous study, maize suspension-cultured cells habituated to low levels of DCB (1.5  $\mu\text{M}$ , Sh1.5) were fed with [ $^3\text{H}$ ]arabinose (de Castro et al. 2015). The feeding experiments demonstrated that these cells had a less efficient and slower metabolism in all phases of the culture cycle, as well as an altered cellular fate of polysaccharides, with a lower amount of strongly wall bound  $^3\text{H}$ -hemicelluloses and an increased presence of soluble extracellular [ $^3\text{H}$ ]-polymers ([ $^3\text{H}$ ]SEPs). One possible explanation for this observation is that as Sh1.5 cells exhibited a reduced cellulose content, which would lead to a decrease in “binding points” for hemicelluloses. Nevertheless, another possibility also emerged, namely that Sh1.5 cells would have a diminished capacity to incorporate

polysaccharides via phenolic cross-linking into the cell wall. This latter alternative is particularly interesting since an increased cross-linking of arabinoxylans leading to higher weight-average relative molecular mass ( $M_w$ ) of these molecules has been shown to be crucial in the habituation of cells to high levels of DCB (Mélida et al. 2009, 2011).

Thus, an extensive analysis was performed in the present study of the relative molecular mass ( $M_r$ ) distribution of hemicelluloses and polymers as well as the phenolic metabolism in several cell compartments of Snh and mildly cellulose-deficient maize cell suspension cultures (Sh1.5) at the early and late logarithmic phases of the culture cycle. To this end, a pulse-chase radio-labelling experimental approach was employed, which consisted of feeding both cell lines with the corresponding radio-labelled metabolic precursors ( $[^3\text{H}]$ arabinose and  $[^{14}\text{C}]$ cinnamic acid respectively). The results obtained in this work contribute to enhancing our understanding of hemicellulose metabolism, providing new clues which help clarify the mechanisms involved in the structural adaptability shown by primary cell walls when exposed to environmental constraints.

## RESULTS

### $M_w$ and $M_r$ distribution of $^3\text{H}$ -polysaccharides

In general, Sh1.5 cells presented more homogeneously sized populations than those from Snh, most of them eluting at an intermediate  $K_{av}$  and therefore having a lower  $M_w$  (Figure 1). In addition, the proportion of  $^3\text{H}$ -polymers and  $^3\text{H}$ -hemicelluloses that were eluted in the V0 was lower in Sh1.5 cells.  $^3\text{H}$ -polysaccharides from Sh1.5 cells had a lower  $M_w$  than those of Snh in all cell compartments and culture phases analysed, with the sole exception of those observed within the protoplasm at late logarithmic phase (Figure 1).

Protoplasmic  $^3\text{H}$ -polymers consisted of a highly poly-disperse population of molecules, which in both cell lines showed a lower  $M_w$  when compared with the cell wall bound  $^3\text{H}$ -hemicelluloses or  $[^3\text{H}]$ SEPs. This result indicates that they underwent massive cross-linking once incorporated into the cell wall or sloughed into the medium. In line with this, when both cell lines were compared, Snh cells showed a higher  $M_w$  and therefore a much more pronounced degree of crosslinking than Sh1.5 cells, independently of the culture phase considered (Figure 1).

The general elution profiles of  $[^3\text{H}]$ SEPs showed that those from Sh1.5 cells were almost identical in both culture phases, whereas in Snh cells a greater proportion of them eluted in

the V<sub>0</sub> at the early logarithmic phase and a lower K<sub>av</sub> was detected (Figure 1). This observation was confirmed by the M<sub>w</sub> data, in which the M<sub>w</sub> of [<sup>3</sup>H]SEPs did not vary depending on the culture phase in Sh1.5 cells, whereas it decreased in the Snh cell line at the late logarithmic phase (Figure 1). In addition, [<sup>3</sup>H]SEPs from Sh1.5 cells had a lower M<sub>w</sub> than Snh ones in both culture phases.

Both types of <sup>3</sup>H-hemicelluloses (0.1 M or 6 M NaOH-extracted) showed a lower M<sub>w</sub> in Sh1.5 cells (Figure 1). In Snh cells, the M<sub>w</sub> of 0.1 M NaOH-extracted wall bound <sup>3</sup>H-hemicelluloses did not vary according to the culture phase whereas it appeared to decrease at the late logarithmic phase in Sh1.5 cells (Figure 2). Likewise, the general elution profile for this type of <sup>3</sup>H-hemicellulose was very similar in the case of Snh cells in both culture phases, whereas Sh1.5 cells showed a slightly greater proportion of <sup>3</sup>H-molecules eluting in a higher K<sub>av</sub> at the late logarithmic phase (Figure 1). In the case of 6 M NaOH-extracted <sup>3</sup>H-hemicelluloses, their M<sub>w</sub> barely increased in Snh cells, whereas in Sh1.5 cells it once again decreased. These findings were repeated in the general elution profiles, where an increase in the proportion of <sup>3</sup>H detected at a lower K<sub>av</sub> was observed at the late logarithmic phase in Snh cells, whereas it decreased in Sh1.5 cells (Figure 1).

#### **M<sub>r</sub> distribution of <sup>3</sup>H-labelled diagnostic fragments from <sup>3</sup>H-polysaccharides**

After Driselase digestion, the elution profiles of polymers containing [<sup>3</sup>H]arabinose, [<sup>3</sup>H]xylose or [<sup>3</sup>H]xylobiose were similar to the general ones, indicating the solidity of the experimental approach (Figures 2, 3 and 4 vs Figure 1). In general terms, elution profiles of polymers containing [<sup>3</sup>H]arabinose, [<sup>3</sup>H]xylose or [<sup>3</sup>H]xylobiose showed similar M<sub>r</sub> distributions. This finding suggests that these diagnostic fragments were a digestion product of a unique type of polysaccharide, putatively [<sup>3</sup>H]arabinoxylan. Reinforcing this idea was the disparity observed between these, and fragments from polymers which contained [<sup>3</sup>H]isoprimeverose, a diagnostic fragment for [<sup>3</sup>H]xyloglucan. Undigested <sup>3</sup>H-polymers were found in all digested fractions, most of them eluting in a K<sub>av</sub> ranging from 0 to 0.5 (Figures 2, 3 and 4).

#### M<sub>r</sub> distribution of 0.1 M NaOH-extracted hemicelluloses diagnostic fragments

The elution profiles of polymers containing [<sup>3</sup>H]arabinose residues and those characteristic for [<sup>3</sup>H]xylans ([<sup>3</sup>H]xylose and [<sup>3</sup>H]xylobiose) were very similar in both cell lines and culture stages. However, the elution profiles of [<sup>3</sup>H]isoprimeverose (diagnostic for [<sup>3</sup>H]xyloglucan) differed from those characteristic of [<sup>3</sup>H]xylans and [<sup>3</sup>H]arabinose-containing polymers (Figure 2 ). M<sub>r</sub> distributions of [<sup>3</sup>H]arabinose-containing polymers and <sup>3</sup>H-labelled diagnostic fragments for xylans in both culture phases, and those for undigested <sup>3</sup>H-polymers at the early logarithmic phase, were very similar to the general elution profile shown in Figure 1.

M<sub>r</sub> distributions of [<sup>3</sup>H]arabinose residues, [<sup>3</sup>H]xylose and [<sup>3</sup>H]xylobiose from Snh cells did not vary substantially according to culture phase. In the case of Sh1.5 cells, the elution profiles for these diagnostic fragments as well as [<sup>3</sup>H]isoprimeverose for [<sup>3</sup>H]xyloglucan showed a slightly greater proportion of molecules with a low M<sub>w</sub> (eluting Kav ranging from 0.5 to 1) at the late logarithmic phase of growth. These observations are in agreement with the results of the estimated M<sub>w</sub> shown in Figure 1.

#### M<sub>r</sub> distribution of 6 M NaOH-extracted hemicelluloses diagnostic fragments

In Snh cells, the elution profiles of polymers containing [<sup>3</sup>H]arabinose residues and those for [<sup>3</sup>H]xylans ([<sup>3</sup>H]xylose and [<sup>3</sup>H]xylobiose) and [<sup>3</sup>H]xyloglucan ([<sup>3</sup>H]isoprimeverose) were very similar in both culture stages (Figure 3). In contrast, the elution profile of [<sup>3</sup>H]xyloglucan from Sh1.5 cells was different from that characteristic for [<sup>3</sup>H]xylans. The undigested <sup>3</sup>H-polymers profile at early logarithmic phase was more similar to [<sup>3</sup>H]xyloglucan, and at late logarithmic phase to polymers containing [<sup>3</sup>H]arabinose, [<sup>3</sup>H]xylose and [<sup>3</sup>H]xylobiose. In both cell lines, the M<sub>r</sub> distribution of polymers with [<sup>3</sup>H]arabinose residues and <sup>3</sup>H-labelled diagnostic fragments for xylans mimicked the general elution profile of 6 M NaOH-extracted hemicelluloses (Figure 3 vs Figure 1).

Most of the polymers which contained [<sup>3</sup>H]arabinose, [<sup>3</sup>H]xylose and [<sup>3</sup>H]xylobiose as well as [<sup>3</sup>H]isoprimeverose and undigested <sup>3</sup>H-polymers extracted from both cell lines, eluted in a Kav ranging from 0 to 0.4 in both culture phases. In addition, the proportion of <sup>3</sup>H detected at the V0 was low in both cell lines and culture phases (Figure 3). Nevertheless, a remarkable difference was found in the elution profile of [<sup>3</sup>H]xyloglucan and undigested



<sup>3</sup>H-polymers from Sh1.5 cells, in which a marked elution peak was observed near a Kav of 0.1 at early logarithmic phase of culture. This elution peak was not appreciable at late logarithmic phase of culture.

#### M<sub>r</sub> distribution of SEPs

In both cell lines, the elution profiles of [<sup>3</sup>H]arabinose, [<sup>3</sup>H]xylose or [<sup>3</sup>H]xylobiose-containing polymers were very similar (Figure 4 vs Figure 1), suggesting that [<sup>3</sup>H]SEPs are mainly constituted by arabinoxylans sloughed into the culture medium. A greater proportion of <sup>3</sup>H-molecules with a slightly higher M<sub>w</sub> at early logarithmic phase was detected in both cell lines when compared with the late logarithmic stage of the culture cycle (Figure 4).

In Snh cells, the elution profiles of the different diagnostic fragments at early logarithmic phase were similar, mainly eluting at a Kav ranging from 0 to 0.5 and at the V0 (Figure 4A). However, M<sub>r</sub> distribution of the undigested <sup>3</sup>H-polymers and the polymers from which [<sup>3</sup>H]xylobiose was released showed a higher degree of similarity between them than with the remaining profiles.

At late logarithmic phase, Snh elution profiles differed from those observed at the early logarithmic culture stage described above (Figure 4B). The elution profiles of [<sup>3</sup>H]xylans, ([<sup>3</sup>H]xylose and [<sup>3</sup>H]xylobiose diagnostic fragments) were similar, and molecules mainly eluted in a Kav ranging from 0.1 to 0.4. Similarly, the elution profiles of [<sup>3</sup>H]xyloglucan ([<sup>3</sup>H]isoprimeverose) and undigested <sup>3</sup>H-polymers were alike. Surprisingly, the elution profile of <sup>3</sup>H-polymers which contained [<sup>3</sup>H]arabinose residues was for the first time different to those for [<sup>3</sup>H]xylans, and showed a wide distribution among the different Kav, with two well-defined “elution areas”. The first of these, in a Kav from 0.2 to 0.4, coincided with that observed for the [<sup>3</sup>H]xylan diagnostic fragments, suggesting a presumable sloughing of arabinoxylans. However, the second elution area was detected in a Kav ranging from 0.5 to 0.8, an observation that would indicate the release into the culture medium of another type of <sup>3</sup>H-molecule population containing [<sup>3</sup>H]arabinose residues. This was not observed in the Sh1.5 cells, in which the elution profile of polymers containing [<sup>3</sup>H]arabinose residues was very similar to those for [<sup>3</sup>H]xylans.

In Sh1.5 cells, the digestion of [<sup>3</sup>H]SEPs with Driselase showed that all the <sup>3</sup>H-polymers (those which contained [<sup>3</sup>H]arabinose, those with diagnostic fragments for [<sup>3</sup>H]xylans and

[<sup>3</sup>H]xyloglucan and the undigested <sup>3</sup>H-polymers) eluted in a higher Kav than those from Snh cells, and therefore had a lower Mw (Figure 4). This was especially clear at the early logarithmic phase (Figure 4A). The most remarkable difference was found in the elution profile of [<sup>3</sup>H]xyloglucan and undigested <sup>3</sup>H-polymers at late logarithmic culture phase (Figure 4B), where a marked peak was observed close to a Kav of 0.3, similar to that previously described for the elution profile of 6 M NaOH-extracted [<sup>3</sup>H]xyloglucan at early logarithmic phase.

## **Analysis of phenolic metabolism**

### Uptake of [<sup>14</sup>C]cinnamic acid from the culture medium and its incorporation into cell compartments

No differences in [<sup>14</sup>C]cinnamic acid consumption were observed among maize cell lines or culture phases (Figure 5). In general, consumption percentages of [<sup>14</sup>C]cinnamic acid ranged between 40%-55% of the total [<sup>14</sup>C]cinnamic acid added at time zero. Most of the [<sup>14</sup>C]cinnamic acid consumption (90%) occurred during the first 15 min after [<sup>14</sup>C]cinnamic acid was fed.

The kinetics of [<sup>14</sup>C]cinnamic acid incorporation into the protoplasmic fraction and cell wall compartment did not show major differences between either cell lines or culture phases, and as it was expected, its incorporation into the protoplasmic fraction preceded its incorporation into the cell wall. However, relative incorporation of [<sup>14</sup>C]cinnamic acid into the Sh1.5 protoplasmic fraction and cell wall was, on average, half-fold reduced when compared with Snh cells (Figure 5).

### [<sup>14</sup>C]cinnamic acid metabolism on the protoplasmic fraction

Hydroxycinnamate <sup>14</sup>C-labelled metabolites incorporated into the protoplasmic fraction of Snh and Sh1.5 cells in their early logarithmic phase (Figures 6A and S1), were tracked during a 180 min pulse of [<sup>14</sup>C]cinnamic acid. In both cell lines, [<sup>14</sup>C]cinnamic acid was quickly metabolised within the first 5 min after its addition to the culture medium. As expected, [<sup>14</sup>C]cinnamic acid was rapidly converted to *p*-[<sup>14</sup>C]coumaric acid and then to [<sup>14</sup>C]ferulic acid. Both *p*-[<sup>14</sup>C]coumaric and [<sup>14</sup>C]ferulic acid, were only transiently accumulated in the protoplasm of Snh cells as their pools largely disappear after 180 min. However, protoplasmic fraction from Sh1.5 cells was enriched in *p*-[<sup>14</sup>C]coumaric and [<sup>14</sup>C]ferulic acid when compared with Snh cells. Rf0 and low-Rf material ([<sup>14</sup>C]dimers and

oligomers) was rapidly detected (5 min) after [ $^{14}\text{C}$ ]cinnamic acid feeding and gradually accumulated at the same extent in Snh and Sh1.5 cells. In both cell lines, the addition of  $\text{H}_2\text{O}_2$  increased the extent of dimerization or oligomerisation.

Kinetics of [ $^{14}\text{C}$ ]cinnamic metabolism did not substantially change when maize suspension cultured-cells in their late logarithmic phase were assayed (Figures 6B and S2). As major differences, a delay in the metabolism of [ $^{14}\text{C}$ ]cinnamic acid by Sh1.5 cells, and a higher accumulation of Rf0 and low-Rf material in Snh cells can be pointed out. Finally, the addition of  $\text{H}_2\text{O}_2$  during this culture phase did not related with an increase of dimerization or oligomerisation.

#### [ $^{14}\text{C}$ ]cinnamic acid metabolism in the cell wall

An analysis of hydroxycinnamate  $^{14}\text{C}$ -labelled metabolites incorporated into the cell wall during early-logarithmic growth phase is shown in Figures 7A and S3. The [ $^{14}\text{C}$ ]cinnamic acid added to the culture medium was rapidly (1-3 min) metabolised to *p*-[ $^{14}\text{C}$ ]coumaric acid and then incorporated into the cell wall. In both cell lines, a decrease was observed in the relative incorporation of *p*-[ $^{14}\text{C}$ ]coumaric acid into the cell wall the first 30 min after [ $^{14}\text{C}$ ]cinnamic acid feeding. Concomitantly the [ $^{14}\text{C}$ ]ferulic acid esterified into the cell wall, abruptly increasing until reaching a plateau phase. Furthermore, at 30 to 60 min after [ $^{14}\text{C}$ ]cinnamic acid feeding a decrease in the amount of cell wall-esterified [ $^{14}\text{C}$ ]ferulic acid was detected in Snh cells.

In all cases, [ $^{14}\text{C}$ ]ferulic acid was the main cell wall hydroxycinnamate detected. The relative quantification of [ $^{14}\text{C}$ ]cinnamic acid [ $^{14}\text{C}$ ]derivatives indicated that Sh1.5 cell walls were enriched in *p*-[ $^{14}\text{C}$ ]coumaric acid and impoverished in [ $^{14}\text{C}$ ]ferulic acid in comparison with Snh cell walls (Figure 7).  $^{14}\text{C}$ -diferuloyl groups and  $^{14}\text{C}$ -oligomers (compounds that migrate at low RF regions) were detected in both cell lines 30-60 min after [ $^{14}\text{C}$ ]cinnamic acid feeding, depending on the cell culture phase. The increase in  $^{14}\text{C}$ -diferulates and  $^{14}\text{C}$ -oligomers occurred concurrently with a decrease in the amount of [ $^{14}\text{C}$ ]ferulic acid. In addition, their relative quantification indicated that [ $^{14}\text{C}$ ]ferulic acid,  $^{14}\text{C}$ -dimers and  $^{14}\text{C}$ -oligomers were more abundant in Sh1.5 cell walls. Lastly, and also at the early logarithmic phase, the supply of  $\text{H}_2\text{O}_2$  provided 120 min after [ $^{14}\text{C}$ ]cinnamic acid feeding induced an increase in the dimerisation or oligomerisation of [ $^{14}\text{C}$ ]ferulate, occurring to a similar degree in both cell lines (Figure 7).

No differences were found in the kinetics of Snh cell wall *p*-coumaroylation and feruloylation between growth phases (Figures 7A and S3 vs 7B and S4). However, some differences were observed in the case of habituated cells: Sh1.5 cell walls accumulated more *p*-[<sup>14</sup>C]coumaric acid, <sup>14</sup>C-dimers and <sup>14</sup>C-oligomers at the late logarithmic growth phase (Figures 7B and S4). Accordingly, the relative decrease in [<sup>14</sup>C]ferulic acid amount was more pronounced in the late logarithmic than in the early logarithmic growth phase. Lastly, the addition of H<sub>2</sub>O<sub>2</sub> did not increase the level of [<sup>14</sup>C]ferulate dimerisation or oligomerisation.

### **Arabidopsis homologue *IRX* gene expression analysis**

The relative expression of the maize genes *GRMZM2G100143*, *GRMZM2G059825* and *gbIBT036881.1*, homologues of arabidopsis *IRX10*, *IRX10-L* and *IRX9*, respectively, was analysed by RT-PCR, shown in Figure S5. In Sh6 cells, the expression of arabidopsis *IRX10* and *IRX9* homologues was induced, whereas a decrease was detected in the expression of the arabidopsis *IRX10-L* homologue when compared with Snh cells. In Sh1.5 cells, a reduced expression of the arabidopsis *IRX10* homologue was observed.

## **DISCUSSION**

It has previously been reported that DCB-habituated maize cells constitute a suitable cellular model to investigate heteroxylan metabolism, since their coping strategies are based on altered arabinoxylan networks which show a certain degree of response plasticity depending on the habituation level (Mélida et al. 2009; de Castro et al. 2014). Likewise, previous studies on maize cells habituated to high DCB levels (12 µM, Sh12) have described compensating strategies based on a reinforced hemicellulosic network acquired by more cross-linked arabinoxylans with a higher M<sub>w</sub> and lower extractability (Mélida et al. 2009, 2011). These results are in contrast to those observed in maize cell suspensions habituated to low DCB levels (Sh1.5), in which the feeding of [<sup>3</sup>H]arabinose revealed a lower proportion of strongly wall-bound <sup>3</sup>H-hemicelluloses and an increase in [<sup>3</sup>H]SEPs, as well as a slower and less efficient metabolism of polysaccharides throughout the entire culture cycle (de Castro et al. 2015).

This finding was especially surprising, and two possible explanations arise to explain this altered cellular fate: it may be caused by 1) a reduction in “binding points” to cellulose and/or 2) a

reduced capacity to incorporate arabinoxylans into the cell wall via extra-protoplasmic phenolic cross-linking.

As cross-linking is closely related to the  $M_w$  of polysaccharides and to phenolic metabolism, both were investigated in the present study, in several cell compartments of Sh1.5 cells throughout the culture cycle. This was achieved through *in vivo* feeding experiments with radio-labelled precursors ( $[^3\text{H}]$ arabinose and  $[^{14}\text{C}]$ cinnamic acid, respectively), since the use of this methodology has been successful in comparable experimental studies (Fry et al. 2000; Kerr and Fry 2003; Burr and Fry 2009; Mérida et al. 2011; de Castro et al. 2015).

The first question emerged was whether low DCB habituation levels entailed modifications in the  $M_w$  of hemicelluloses and polymers. In general, Sh1.5 cells showed more homogeneously sized and smaller  $^3\text{H}$ -polysaccharides populations that were more similar among cell compartments, regardless of the culture phase, than those from Snh. These results, and the suitability of the experimental approach, were supported by the observation that these trends were consistent in the elution profiles of the different diagnostic fragments (unique for  $[^3\text{H}]$ xyloglucan,  $[^3\text{H}]$ xylans, polymers which containing  $[^3\text{H}]$ arabinose and those undigested by Driselase) and were perfectly illustrated in the case of  $[^3\text{H}]$ SEPs. These latter polymers are sloughed into the extracellular medium and no extraction treatment is required in order to obtain them; their molecular integrity is maintained intact. Therefore, the data obtained from the study of  $[^3\text{H}]$ SEPs are especially valuable providing information about features of polysaccharides in their native structure (Kerr and Fry 2003). In addition, some enzymes, substrates such as  $\text{H}_2\text{O}_2$  and inhibitors of cross-linking (Encina and Fry 2005), also diffuse into the extracellular medium (Burr and Fry 2009).  $^3\text{H}$ -polysaccharides from Sh1.5 cells also had a lower  $M_w$  in all cell compartments, independently of the culture cycle stage. A comparison of the  $M_w$  from protoplasmic  $^3\text{H}$ -polymers and cell wall bound  $^3\text{H}$ -hemicelluloses indicated that after secretion into the cell wall, protoplasmic  $^3\text{H}$ -polymers underwent massive cross-linking in both cell lines, although this increase was much less pronounced in Sh1.5 cells. These results point to a reduction in the cell wall grafting ability of Sh1.5 cells.

A possible explanation for these findings is that cells habituated to low DCB levels synthesized  $^3\text{H}$ -molecules with a lower and similar  $M_w$  as a strategy to cope with the lack of cellulose, considered as “mild” when compared with that observed in cells habituated to high DCB levels (33% vs 75% of reduction compared with Snh). The pattern observed in

the expression of arabidopsis *IRX* orthologue genes in habituated cells (Sh1.5 and Sh6) supports this hypothesis. As mentioned above, cells habituated to high DCB levels synthesized hemicelluloses with a higher  $M_w$  in order to counteract their impoverishment in cellulose. In good agreement with this, the expression of genes involved in the xylan elongation chain (Brown et al. 2009; Wu et al. 2009) *GRMZM2G100143* orthologue of *AtIRX10* as well as *gbIBT036881.1*, the orthologue of *AtIRX9* (Bosch et al. 2011) involved in the initiation of the xylan synthesis (Brown et al. 2007; Pena et al. 2007) was found to be up-regulated in Sh6 cells. Interestingly, in cells habituated to low-DCB levels (Sh1.5), *GRMZM2G100143* (*AtIRX10* orthologue) was found to be repressed whereas the expression of *gbIBT036881.1* (*AtIRX9* orthologue) remained unaltered. Such preliminary results may indicate that Sh1.5 cells do not in fact present a defective initiation of xylan synthesis but are impaired in the elongation of hemicellulose chains. This would lead to the production of shorter xylan chains in Sh1.5 cells, contrary to what happens in Sh6 (Supplementary data Figure S5).

In addition, a previous study on maize cells habituated to medium and high DCB levels revealed differences in the  $M_w$  of wall-bound hemicelluloses (Mélida et al. 2009). In this study, polysaccharides were subsequently extracted from the cell wall using two alkali-treatments, 0.1 M KOH and 4 M KOH, known to solubilise weakly- and firmly-bound polysaccharide populations respectively. The results showed that in medium DCB-habitation levels, the  $M_w$  of 0.1 M KOH-extracted hemicelluloses increased with respect to the same fraction obtained from non-habituating cells, cells habituated to high DCB levels, and even with respect to 4 M KOH-extracted hemicelluloses. Interestingly, in maize cells habituated to high DCB levels, the  $M_w$  of 4 M KOH-extracted hemicelluloses increased with respect to non-habituating cells, cells habituated to medium DCB levels and to those solubilised by 0.1 M KOH treatment. It is probable that, as the level of DCB habituation increases, a progressive input of higher  $M_w$  hemicelluloses that are more tightly bound takes place in order to reinforce the cell walls. However, it should be borne in mind that these results were obtained using a different experimental approach to ours. The maize cells used in Mélida et al. (2009) were collected at an advanced stage of the culture cycle. Thus, it is feasible to assume that hemicelluloses in such walls have been there for a longer period of time and have presumably been subjected to grafting processes with other molecules, which would lead to an increase in their  $M_w$ . Furthermore, it is important to note that massive cross-linking of hemicelluloses in maize cell suspension cultures occurred 11-days after

sub-culturing (Burr and Fry 2009). In contrast, our experimental approach involved a pulse-chase experiment, in which the screened molecules were formed, integrated into the wall or sloughed over a short period of time (5 h). Despite this, the idea that maize cells gradually require less extractable and higher  $M_w$  hemicelluloses as the DCB habituation level increases is in good agreement with observations in previous studies, where cells habituated to low and medium DCB levels seemed to compensate for their lack in cellulose through an increase in hemicelluloses extracted with mild alkali treatment (0.1 M KOH) (Mélida et al. 2009; de Castro et al. 2014). The finding that Sh1.5 cells had molecules, and more specifically hemicelluloses, with a lower  $M_w$  than those from Snh may indicate that cells habituated to low DCB levels increase the amount of low  $M_w$  hemicelluloses as a part of their coping strategy. In this respect, it has previously been suggested that a decrease in the  $M_w$  of xyloglucan improves its capacity to bind to cellulose (Lima et al. 2004), and in accordance with this, it has been found that xyloglucan from cellulose-deficient bean cells has a lower  $M_w$  than that of control cells (Alonso-Simón et al. 2007). In this latter case, it was hypothesised that smaller xyloglucan molecules together with an increased xyloglucan-endo-transglucosylase activity would produce a more rigidified cell wall (Alonso-Simón et al. 2007). However, it should be borne in mind that this finding was described in bean cells, which have a type I cell wall. Therefore, it still remains unclear whether this mechanism could play any kind of role in cells with a type II cell wall.

Another possible explanation is closely related to the capacity of Sh1.5 cells to cross-link cell wall hemicelluloses and SEPs. As previously mentioned, Sh1.5 cells showed a reduced amount of cell wall bound  $^3\text{H}$ -hemicelluloses and an increased presence of  $^3\text{H}$ SEPs in the culture medium (de Castro et al. 2015). In addition, the timing of cross-linking may also be delayed in Sh1.5 cells due to their slower and less efficient metabolism (de Castro et al. 2015) thus preventing detection during the time window employed in this experiment (5 h). In order to explore these possibilities, an analysis was performed of cinnamic acid metabolism as well as a characterization of its derivatives on protoplasmic fraction and in the cell wall. First, no marked differences were detected in the capacity to uptake  $^{14}\text{C}$ cinnamic acid from the culture medium depending on the cell line, a finding that it is in agreement with observations in a previous study on maize cells habituated to high DCB levels and employing a similar experimental approach (Mélida et al. 2011). Nevertheless, Snh and Sh1.5 cells showed differences in their capacity to incorporate  $^{14}\text{C}$ cinnamic acid into the protoplasm and cell wall; habituated cells presented a deficient capacity for the

414 biosynthesis and incorporation into the cell wall of [<sup>14</sup>C]feruloylated molecules. Similar  
415 altered kinetics in Sh1.5 cells were observed when [<sup>3</sup>H]arabinose was fed as the radioactive  
416 precursor, showing that a delayed cellular traffic and cell wall binding of <sup>3</sup>H-hemicelluloses  
417 and <sup>3</sup>H-polymers took place (de Castro et al. 2015). However, and despite this reduced  
418 capacity, the relative content in ferulate dimers and oligomers was higher in Sh1.5 cells.  
419 These results are in accordance with those previously observed in maize cells habituated to  
420 high DCB levels (Mélida et al. 2010b, 2011) supporting the hypothesis that a greater cross-  
421 linked network of hemicelluloses, mainly arabinoxylans, would be crucial for the cell wall  
422 reinforcing strategy in DCB-habituated maize cells. Thus, the results suggest that the lower  
423 M<sub>w</sub> of hemicelluloses and polymers detected in Sh1.5 cells was not related to a lower degree  
424 of cross-linkage through ferulic acid. Alternatively, hemicelluloses and polymers from  
425 habituated cells were in fact shorter, albeit extensively cross-linked.

426 Another possibility could be that dimerisation (or oligomerisation) of ferulic acid residues  
427 contributed to the formation of intra-polymeric loops to a greater extent (Fry et al. 2000).  
428 Consequently, Sh1.5 hemicelluloses would be deposited on the cell wall as a coagulum.  
429 However, a negative association between intra-polymeric arabinoxylan cross-linking and  
430 cell wall reinforcement has been suggested (Fry et al. 2000). Consequently, this latter  
431 finding would render this possibility unlikely in the scenario of cell wall rigidification.

432 Changes in the M<sub>w</sub> of maize cell wall hemicelluloses and polymers during the culture cycle  
433 have been demonstrated, which is not surprising since cells would need to modify them in  
434 order to allow or restrict cell expansion (Kerr and Fry 2003). In addition, ferulate cross-  
435 linking in maize cells has also been shown to vary throughout the culture cycle, according  
436 to the physiological condition of the cells (Burr and Fry 2009). In this respect, and as a  
437 precedent, differences in the phenolic profile and metabolism of cells habituated to high  
438 DCB levels have previously been reported as the culture cycle progresses (Mélida et al.  
439 2011). Thus, the study of how the M<sub>w</sub> of hemicelluloses and polymers as well as the phenolic  
440 metabolism vary during the culture cycle would be of interest in the case of maize cells  
441 habituated to low DCB levels, which have been demonstrated to present altered patterns and  
442 kinetics of growth (de Castro et al. 2014), as well as a delayed metabolism throughout the  
443 entire cycle (de Castro et al. 2015).

444 Generally, the elution profiles of Driselase-digested diagnostic fragments for polymers  
445 containing [<sup>3</sup>H]arabinose and those for [<sup>3</sup>H]xylans were very similar, although arabinose



residues can derive from other polymers besides arabinoxylans, such as rhamnogalacturonan type I, or arabinogalactan proteins. However, a sugar analysis of 0.1 M- and 6 M alkali-extracted fractions from Sh1.5 cell walls revealed a low content of uronic acid, galactose and rhamnose residues (de Castro et al. 2014). Therefore, it can be assumed that [<sup>3</sup>H]arabinose, [<sup>3</sup>H]xylose and [<sup>3</sup>H]xylobiose are, in fact, digestion products of putative [<sup>3</sup>H]arabinoxylan molecules.

In both Snh and Sh1.5 cell lines, a greater proportion of smaller molecule populations were detected at the late logarithmic phase compared with the early logarithmic phase. In general, this trend was observed for arabinoxylans, xyloglucan and Driselase-undigested elution profiles regardless of the cell compartment, and was confirmed by the data from the  $M_w$  calculation. Some of the main factors controlling ferulate cross-linking (and therefore involved in changes in the  $M_w$  of hemicelluloses), such as peroxidase action and H<sub>2</sub>O<sub>2</sub> availability, varied during the culture cycle (Burr and Fry 2009). Thus, it is feasible that changes in these factors were responsible for the lower  $M_w$  detected at the late logarithmic phase of culture in both cell lines.

The increased  $M_w$  of SEPs compared with cell wall bound hemicelluloses from Snh at the early logarithmic phase may indicate that polysaccharides sloughed into the culture medium underwent additional cross-linking. Indeed, SEPs differ from wall bound hemicelluloses in having greater conformational freedom and mobility, rendering them more accessible to enzymes, which would increase the degree of ferulate polymerisation (Burr and Fry 2009). Nevertheless, the trend described was not observed either in Snh cells at late-logarithmic phase or in Sh1.5 cells independently of the cell culture phase.

Snh cells shown faster [<sup>14</sup>C]cinnamic acid metabolism than Sh1.5 and as a consequence free-phenolics and <sup>14</sup>C-labelled compounds were less persistent on the protoplasmic fraction. However the dimerization and oligomerization process were quickly in Sh1.5, especially during the late logarithmic phase. The presence of dimers and oligomers in the protoplasm of Sh1.5 cells, could be related with the  $M_w$  increase of the polymers (lower  $K_{av}$ ) of this fraction at late logarithmic phase compared with early logarithmic phase. Additionally, in the cell wall compartment, the  $M_w$  of polysaccharides in Snh cells was very similar in both culture stages, and the same trend of similarity between culture phases was observed in Snh kinetics of cell wall *p*-coumaroylation and feruloylation. In contrast, the  $M_w$  of Sh1.5 cell wall hemicelluloses and polymers decreased in the late logarithmic phase,

but surprisingly, greater amount of ferulate  $^{14}\text{C}$ -dimers and  $^{14}\text{C}$ -oligomers were detected. In line with this, exogenous  $\text{H}_2\text{O}_2$  was observed to stimulate feruloyl dimerisation in Snh cells independently of the growth phase, suggesting  $\text{H}_2\text{O}_2$  availability as the limiting factor for ferulate dimerisation and oligomerisation in the case of Snh cells. In contrast, since exogenous  $\text{H}_2\text{O}_2$  in Sh1.5 cells at the late logarithmic growth phase did not produce any change, the limiting factor seems to be the availability of free ferulate for an extra dimerisation. This latter finding once again demonstrated that hemicelluloses and polymers from habituated cells would in fact be shorter, albeit widely cross-linked, and/or underwent an additional trimming process that did not occur in Snh cells.

Another interesting finding was the marked elution peak in the Sh1.5 xyloglucan molecules extracted with 6 M NaOH as well as in those sloughed into the culture medium observed at  $K_{av}$  0.1 and 0.3 respectively. A very high  $M_w$  of wall bound xyloglucan molecules in maize suspension cells has previously been reported, suggesting that a high  $M_w$  complex of xyloglucan, possibly linked to other polysaccharides, may play a more effective tethering role. This finding would partially explain why graminaceous monocots can survive with much smaller quantities of xyloglucan than dicots (Kerr and Fry 2003). Indeed, neither of the marked elution peaks of Sh1.5 xyloglucan was detected in the V0, where molecules with the highest  $M_w$  would elute. However, when both  $K_{av}$  were compared, strongly wall bound xyloglucan from Sh1.5 cells was detected in a lower  $K_{av}$  than that for the sloughed xyloglucan (0.1 vs 0.3). This would in fact indicate that  $M_w$  increased in wall bound xyloglucan in Sh1.5, probably through an association with other polysaccharides or polymers. Since it has previously been suggested that xyloglucan could be involved in the coping response induced at low DCB levels (de Castro et al. 2014), these results would partially support this hypothesis, although further experiments are required for confirmation.

In sum, the study of the  $M_w$  of  $[^3\text{H}]$ xylans,  $[^3\text{H}]$ xyloglucans,  $[^3\text{H}]$ arabinose-containing polymers and Driselase-digestion resistant  $^3\text{H}$ -polymers as well as the phenolic metabolism in maize cells habituated to low DCB levels revealed that: a) Sh1.5 cells synthesised molecules with a lower  $M_w$  and a more homogeneous size throughout the entire culture cycle, b) although hemicelluloses were shorter than those observed in the non-habituated cells, this was not related to a diminished capacity for polysaccharide cross-linking, c) both cell lines synthesised molecules with a lower  $M_w$  at the late logarithmic phase of the culture cycle, probably associated with the cessation of growth, and d) the hypothesis of a

significant role for xyloglucan in habituation to low levels of DCB was supported by the results.

Our results indicate that Sh1.5 cells synthesise shorter, albeit extensively cross-linked, molecules as part of their coping strategy. These results demonstrate that DCB habituation is a gradual and dynamic process in which maize cells show highly plastic coping responses depending on the DCB habituation framework or, the equivalent, on the level of cellulose deficiency.

## **MATERIALS AND METHODS**

### **Cell cultures and habituation to DCB**

Maize cell-suspension cultures (*Zea mays* L., Black Mexican sweet corn) from immature embryos were grown in Murashige and Skoog media (Murashige and Skoog 1962) supplemented with 9  $\mu\text{M}$  2,4-D and 20  $\text{g L}^{-1}$  sucrose, at 25°C under light and rotary shaken, and routinely subcultured every 15 days.

In order to obtain cell cultures habituated to DCB, Snh were stepwise subcultured in a medium supplied with increasing concentrations of DCB, dissolved in dimethyl sulfoxide (DMSO) which did not affect cell growth at this range of concentrations. Snh cells were transferred to a medium containing 1  $\mu\text{M}$  DCB, increasing the DCB concentration up to 1.5  $\mu\text{M}$  DCB (Sh1.5) after seven subcultures. Cell suspensions habituated to high DCB levels were derived from maize callus cultures habituated to grow in 12  $\mu\text{M}$  DCB which were transferred into liquid medium supplemented with 6  $\mu\text{M}$  DCB (Sh6). Growth curves of Snh and Sh1.5 maize cell lines were obtained by measuring the increase in dry weight at different culture times and it was observed that Sh1.5 cells presented longer culture phases than Snh cells (de Castro et al. 2014). Therefore, these growth curves were subsequently used to select the most appropriate days for sampling in order to ensure that Snh and Sh1.5 cell lines were at the same stage of the culture cycle.

### **Analysis of $M_r$ distributions**

#### **Radio-labelling of liquid cultures with [ $^3\text{H}$ ]arabinose**

Thirteen-ml aliquots from Snh and Sh1.5 maize cells cultures were collected at early logarithmic and late logarithmic growth phases and each 13-ml aliquot was independently fed with 1 MBq of L-[1-<sup>3</sup>H]arabinose (148 GBq/mmol; Amersham International, Bucks., U.K). The days on which the 13-ml aliquot of culture was sampled and [<sup>3</sup>H]arabinose was added, were adjusted depending on the cell line at early and late logarithmic phases. Thus, for the early logarithmic phase, the 4<sup>th</sup> and 8<sup>th</sup> days of culture were chosen for Snh and Sh1.5 cell lines, respectively (see de Castro et al. 2014). In the case of late logarithmic phase, the 8<sup>th</sup> and 12<sup>th</sup> days were selected for Snh and Sh1.5 cell lines, respectively. In order to obtain an optimal quantity of radio-labelled <sup>3</sup>H-hemicelluloses and <sup>3</sup>H-polymers, samples for analysis of the M<sub>r</sub> were collected at different labelling-time points selected according to the maximum value of incorporated [<sup>3</sup>H] arabinose observed in the kinetics previously described (de Castro et al. 2015). Thus, in the case of protoplasmic polymers, 1-ml samples were collected from independent 13-ml aliquots of culture 30 min and 60 min after feeding [<sup>3</sup>H] arabinose for Snh and Sh1.5 cells, respectively. For the remaining <sup>3</sup>H-hemicelluloses and <sup>3</sup>H-polymers, sampling was carried out 300 min after feeding [<sup>3</sup>H]arabinose for both cell lines.

#### Obtaining <sup>3</sup>H-hemicelluloses and <sup>3</sup>H-polymers from discrete maize cell suspensions compartments

Each 1-ml sample collected at the different labelling-time points was filtered through an empty Poly-Prep column. Then, cells were rinsed with 5 ml of ice-cold fresh medium. The filtrate was pooled with the rinses and labelled as cell-free medium fraction.

The washed cells retained in the Poly-Prep column filter were quickly resuspended in 1 ml of homogenisation buffer [50 mM collidine acetate (pH 7.0) containing 2% (w/v) lithium dodecyl sulfate (LiDS), 10% (v/v) glycerol, 5 mM sodium thiosulphate and 10 mM dithiothreitol (added freshly)] and stored at -20°C in the Poly-Prep column.

The cell suspension was then thawed at 1.5°C and transferred to the 10-ml glass mortar of a Potter–Elvehjem homogeniser by rinsing with 1 ml of chilled homogenisation buffer (x3 times), and then homogenised with a motor-driven Teflon pestle for 10 min. The homogenate was passed through a second empty Poly-Prep column and the filtrate collected and the cell fragments were rinsed with 1 ml H<sub>2</sub>O (x4 times).

Solid KCl was added to the filtrate and rinses to a final concentration of 0.67 M in order to precipitate the dodecyl sulphate as insoluble  $K^+$  salt. After 30 min at 4°C, the products were centrifuged at 3000 rpm for 5 min. The supernatant was removed and re-centrifuged and this procedure was repeated until the supernatant turned clear, moment in which was collected and considered the protoplasmic fraction.

Cell fragments retained in the filter of the Poly-Prep column were treated with 1 ml of 0.1 M NaOH containing 1% (w/v)  $NaBH_4$  for 24 h at room temperature. The filtrate was then collected and the residue washed with 1 ml of the same extractant (x3 times). Filtrate and washes were pooled and labelled as 0.1 M NaOH fraction. The remaining residue was then treated with 6 M NaOH containing 1% (w/v)  $NaBH_4$  for 24 h at 37°C. The filtrate was again collected and the residue washed with 1 ml of the same solution (x3 times). Filtrate and washes were then pooled and labelled as 6 M NaOH fraction. Both the 0.1 M NaOH and 6 M NaOH fractions were acidified to pH 4.7 by the addition of acetic acid.

#### Gel permeation chromatography

Samples of cell-free medium, protoplasmic and cell wall fractions were dialysed against distilled water with 0.1% (w/v) chlorobutanol at 4°C for 72 h, using a 12 kDa cut-off and 1.5 cm diameter dialysis membrane. Then, the dialysed samples were centrifuged for 15 min at 3000 rpm in order to separate the soluble from the insoluble material in water.

To identify the void volume ( $V_0$ ) and the totally included volume ( $V_i$ ), 6 mg dextran (5–40 MDa) and 0.8 mg glucose, respectively, were added to 4 ml of this  $^3H$ -polymer solution as markers. The polymers were then size-fractionated on Sepharose CL-4B (72 ml bed volume in a 1.5-cm-diameter column) in pyridine/acetic acid/water (1/1/23 by vol. pH 4.7 containing 0.5% chlorobutanol) at 12.5 ml/h. Fractions were assayed for total  $^3H$  and the markers were quantified by the anthrone assay (Dische 1962). Estimated recovery of  $^3H$ -hemicelluloses from the Sepharose columns was routinely >80% (Kerr and Fry 2003).

The Sepharose CL-4B column was calibrated with commercial dextrans of known  $M_w$ . Each dextran preparation was run as a mixture with a trace of very high- $M_w$   $^3H$ -hemicellulose from maize culture medium and [ $^{14}C$ ]glucose as internal markers, which defined the  $V_0$  and  $V_i$  ( $K_{av}$  0 and 1) respectively.  $M_w$  was obtained using the  $K_{av(1/2)}$  method (Kerr and Fry 2003) with the calibration curve [ $\log M_w = -3.547 K_{av(1/2)} + 7.2048$ ] obtained for this

column. The  $M_w$  estimates were nominal rather than absolute due to the conformational differences between dextran and hemicelluloses.

## **Characterization of phenolic metabolism**

### $[^{14}\text{C}]$ cinnamic acid synthesis

$[^{14}\text{C}]$ cinnamic acid was prepared from L-[U- $^{14}\text{C}$ ]phenylalanine (Perkin-Elmer; 487 Ci/mol) following the method described by Lindsay and Fry (2008).

### $[^{14}\text{C}]$ cinnamic acid consumption and incorporation into different cell compartments

Fifteen-ml aliquots of Snh and Sh1.5 cell suspensions (20% of settled cell volume) at early and late logarithmic phases of the culture cycle were transferred into 100-ml glass flasks, loosely capped and shaken (150 rpm) for 1 h at 25°C, in order to adapt them to the new environmental conditions. Then,  $[^{14}\text{C}]$ cinnamic acid (0.29  $\mu\text{Ci}$ ) was added to each cell culture. At selected time-points, 400  $\mu\text{l}$  of cell cultures were sampled and acidified by the addition of 15  $\mu\text{l}$  99% formic acid, stopping the consumption and incorporation of  $[^{14}\text{C}]$ cinnamic acid.

Each aliquot sampled at the different labelling-time points was then centrifuged for 4 min at 10000 rpm and the resultant supernatant was collected. Cells were washed with 200  $\mu\text{l}$  of distilled water and subjected again to centrifugation. Supernatant and washings were then pooled, labelled and stored as cell-free medium fraction.

Remaining cells were then incubated for 18 h with 800  $\mu\text{l}$  of 80% ethanol on a rotary wheel, the samples were subsequently centrifuged at 10000 rpm for 4 min and the supernatant was collected. Cells were rinsed with 200  $\mu\text{l}$  of 80% ethanol and centrifuged again. Supernatant and washings were then pooled and labelled as the protoplasmic fraction (alcohol soluble material). The residual cell fragments were considered alcohol insoluble residue (AIR).

### Obtaining of cinnamic acid $[^{14}\text{C}]$ derivatives from discrete maize cell compartments

Four hundred  $\mu$ l-aliquots of Snh and Sh1.5 cell-suspensions at early and late logarithmic phases of the culture cycle were transferred into flat-bottomed vials and shaken (75 rpm) for 1 h at 25°C to allow them to acclimatise to their new environmental conditions. Then, vials were fed with [ $^{14}$ C]cinnamic acid (0.116  $\mu$ Ci). At selected time-points, aliquots were acidified by the addition of 15  $\mu$ l 99% formic acid, stopping [ $^{14}$ C]cinnamic acid incorporation. Where indicated, 40 mM H<sub>2</sub>O<sub>2</sub> (final concentration) was added to some of the samples 120 min after [ $^{14}$ C]cinnamic acid and then shaken for 60 min. Aliquots of cell suspensions contained in the vials were then transferred into Poly-Prep columns and the filtrate was collected and considered the cell-free medium fraction.

The remaining cells were resuspended in 1 ml 80% ethanol and incubated for 18 h on a rotary wheel. Cell suspensions were filtered and the alcohol-soluble fraction was considered the protoplasmic fraction. The protoplasmic fraction was collected, de-esterified with 0.5 M NaOH for 18 h at 25°C, acidified to pH 5.0 and further partitioned against ethyl acetate (x2 times). The ethyl acetate phases were vacuum-dried, re-dissolved in 0.5 ml of acidified water (0.01 N HCl) and further subjected to a second ethyl acetate partition (x3 times). The organic phases were collected, vacuum-dried and re-dissolved in propan-1-ol.

The AIR retained on the Poly-Prep column was de-esterified with 0.5 M NaOH for 18 h at 25°C. The filtrate was collected and the remaining cell fragments were rinsed with 1 ml of distilled water. Filtrate and washings were then pooled, acidified to pH 5.0 and collected as the 0.5 M NaOH fraction. This fraction was subjected to ethyl acetate partition as indicated above. The organic phases were collected, vacuum-dried and re-dissolved in propan-1-ol.

## **Assay of radioactivity**

### Analysis of M<sub>r</sub> distribution

To obtain the general elution profiles of  $^3$ H-hemicelluloses and  $^3$ H-polymers, a 200  $\mu$ l aliquot of column fractions was assayed for radioactivity by liquid scintillation counting in 2 ml of OptiPhase HiSafe (Fisher).

In order to obtain the elution profiles corresponding to  $^3$ H-labelled diagnostic fragments, fractions obtained from the column were pooled in pairs and vacuum-dried. Then, dried samples were subjected to a mild-hydrolysis by re-dissolving them in 500  $\mu$ l of 0.1 M trifluoroacetic acid (TFA), heated at 85°C for 1 h to cleave arabinofuranosyl linkages, and finally re-dried in vacuo to remove TFA. The dried material was re-dissolved in 20  $\mu$ l 0.5%

(w/v) Driselase (partially purified by the method reported by Fry 2000) in pyridine/acetic acid/water (1/1/23 by vol. pH 4.7 containing 0.5% chlorobutanol), and incubated at 37°C for 96 h. The reaction was stopped by the addition of 15% formic acid. The mild acid pretreatment greatly increased the yield of xylose and xylobiose generated during subsequent digestion with Driselase and did not decrease the yield of isoprimeverose (Kerr and Fry 2003). An internal marker mixture containing xylose, arabinose, glucose, isoprimeverose and xylobiose ( $\approx 50$   $\mu\text{g}$  each) was then added to the digest, which was subjected to paper chromatography on Whatman 3MM in ethyl acetate/pyridine/water (9/3/2 by vol.) for 18 h (Thompson and Fry 1997). The internal markers were slightly stained with aniline hydrogen-phthalate and the identified spots were cut out and soaked overnight in 1 ml water [containing 0.5% (w/v) chlorobutanol]. Then, 10 ml of OptiPhase HiSafe scintillation liquid were added, and vials were shaken continuously for 24 h. Radioactivity was subsequently assayed by counting.

#### [ $^{14}\text{C}$ ]cinnamic acid consumption and incorporation into different cell compartments

To analyse the cell-free medium samples and the protoplasmic fraction, 5 and 3 ml of scintillation liquid EcoscintA (National Diagnostics) were added to the sample respectively, and radioactivity was then assayed by counting.

In the case of the radioactivity incorporated into the AIR, cell fragments were firstly resuspended in 0.2 ml of distilled water; 3 ml of scintillation liquid EcoscintA was subsequently added, and the radioactivity was assayed by counting.

#### Obtaining cinnamic acid [ $^{14}\text{C}$ ] derivatives from discrete maize cell compartments

Samples of protoplasmic and AIR fractions de-esterified with 0.5 M NaOH were subjected to thin layer chromatography (TLC). TLC was carried out on plastic-backed silica-gel with a fluorescent indicator (Merck) in benzene/acetic acid (9/1) (v/v). During development, TLC plates were exposed to 312 nm, which maintains hydroxycinnamates as rapidly interconverting single spots of *cis/trans* isomers. Standards of ferulic acid, *p*-coumaric acid and 5-5'-diferulate were used as external markers.



Tracks corresponding to different samples were cut off in pieces of 0.5x1.0 cm or 1.0x1.0 cm from the TLC and analysed by scintillation counting through the addition of 3 ml of scintillation liquid EcoscintA.

#### **Arabidopsis *IRX* homologue gene expression analysis**

Total RNA was extracted with Trizol Reagent (Invitrogen) and reverse-transcribed using the Superscript III first strand synthesis system for RT-PCR (Invitrogen). First-strand cDNA was generated with an oligo(dT)20 primer and used as a template in subsequent PCR reactions. For each assay, several cycles were tested to ensure that amplification was within the exponential range. Ubiquitin was used as housekeeping gene.

Primers (Table S1) were used for the analysis of maize genes *GRMZM2G100143*, *GRMZM2G059825* and *gbIBT036881.1*, homologues of arabidopsis *IRX10* (*AT1G27440*), *IRX10-L* (*AT5G61840*) and *IRX9* (*AT2G37090*), respectively (Bosch et al. 2011). Sequences for *GRMZM2G100143* and *GRMZM2G059825* were derived from the maize genome browser and that for *gbIBT036881.1* from the Phytozome.

#### **ACKNOWLEDGEMENTS**

The authors thank Dr Jesús Álvarez and Dr Asier Largo-Gosens for their helpful scientific discussion, Janice Miller her excellent technical support and Denise Phelps for the English revision of the manuscript. This study was supported by grants from the Spanish Ministry of Science and Innovation (CGL2008-02470 and AGL2011-30545-C02-02) and by the UK Biotechnology and Biological Sciences Research Council (BB/C505791/1). María de Castro and Romina Martínez-Rubio received funding through PhD grants from the Spanish Ministry of Science and Innovation FPI (BES-2009-021688) and FPU (FPU/03505) programs, respectively.

#### **AUTHOR CONTRIBUTIONS**

María de Castro (M.d.C) and Romina Martínez-Rubio (R.M-R) performed most of the research. Stephen C. Fry (S.C.F) supervised and designed the polysaccharides metabolism

experiment which was carried out by M.d.C. Antonio Encina (A.E) supervised and designed the phenolic metabolism experiments. Penélope García-Angulo (P.G-A) and M.d-C drafted the manuscript and made the gene expression analysis. Jose Luis Acebes (J.L.A) provided the research environment and supervised the project. All the authors revised the manuscript. All the work was coordinated and supervised by P. G-A.

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## 821 FIGURES AND TABLES LEGENDS

822 **Figure 1:** Mr distribution profile of  $^3\text{H}$ -polysaccharides: protoplasmic  $^3\text{H}$ -polymers, cell  
823 wall bound  $^3\text{H}$ -hemicelluloses extracted with 0.1 M or 6 M NaOH and soluble extracellular  
824  $^3\text{H}$ -polymers (SEPs) obtained from non-habituated (Snh, purple line) and habituated to 1.5  
825  $\mu\text{M}$  DCB (Sh1.5, green line) maize cell cultures. Data shown are from the early logarithmic  
826 (A) and late logarithmic (B) phase of culture cycle. Samples used were taken 300 minutes  
827 after feeding of [ $^3\text{H}$ ]arabinose, except in the case of protoplasmic  $^3\text{H}$ -polymers, collected  
828 30 and 60 minutes after feeding of [ $^3\text{H}$ ]arabinose for Snh and Sh1.5 respectively.  $^3\text{H}$ -  
829 polymers and  $^3\text{H}$ -hemicelluloses were size-fractionated on Sepharose CL-4B. One cKav is  
830 defined as an interval of 0.01 on the  $x$ -axis. The profiles are scaled such that the area under

the curve is equal (100%) in each frame. Mw values, derived from the median Kav(1/2) of the profiles and then converted to Mw via a calibration curve, are included in the text frame.

**Figure 2:** Mr distribution profile of <sup>3</sup>H-labelled diagnostic fragments from <sup>3</sup>H-polysaccharides: cell wall bound <sup>3</sup>H-hemicelluloses extracted with 0.1 M NaOH obtained from non-habituated (purple line) and habituated to 1.5 μM DCB (green line) maize cell cultures. Data shown are from the early logarithmic (A) and late logarithmic (B) phase of culture cycle. Samples used were taken 300 minutes after feeding of [<sup>3</sup>H]arabinose. Digest of each fraction were assayed for radioactive fragments characteristic of polymers which have arabinose residues {[<sup>3</sup>H]arabinose}, xylans {[<sup>3</sup>H]xylose} and {[<sup>3</sup>H]xylobiose}, xyloglucans {[<sup>3</sup>H]isoprimeverose} and polymers that Driselase does not digest {undigested <sup>3</sup>H-polymers}. Other details as in Figure 1.

**Figure 3:** Mr distribution profile of cell wall bound <sup>3</sup>H-hemicelluloses extracted with 6 M NaOH obtained from non-habituated (purple line) and habituated to 1.5 μM DCB (green line) maize cell cultures. Data shown are from the early logarithmic (A) and late logarithmic (B) phase of culture cycle. Samples used were taken 300 minutes after feeding of [<sup>3</sup>H]arabinose. Digest of each fraction were assayed for radioactive fragments characteristic of polymers which have arabinose residues {[<sup>3</sup>H]arabinose}, xylans {[<sup>3</sup>H]xylose} and {[<sup>3</sup>H]xylobiose}, xyloglucans {[<sup>3</sup>H]isoprimeverose} and polymers that Driselase does not digest {undigested <sup>3</sup>H-polymers}. Other details as in Figure 1.

**Figure 4:** Mr distribution profile of soluble extracellular <sup>3</sup>H-polymers obtained from the culture medium of non-habituated (purple line) and habituated to 1.5 μM DCB (green line) maize cell cultures. Data shown are from the early logarithmic (A) and late logarithmic (B) phase of culture cycle. Samples used were taken 300 minutes after feeding of [<sup>3</sup>H]arabinose. Digest of each fraction were assayed for radioactive fragments characteristic of polymers which have arabinose residues {[<sup>3</sup>H]arabinose}, xylans {[<sup>3</sup>H]xylose} and {[<sup>3</sup>H]xylobiose}, xyloglucans {[<sup>3</sup>H]isoprimeverose} and polymers that Driselase does not digest {undigested <sup>3</sup>H-polymers}. Other details as in Figure 1.

**Figure 5:** Kinetics of consumption and incorporation of radioactivity from exogenous [<sup>14</sup>C]cinnamic acid in non-habituated (purple line) and habituated to 1.5 μM DCB (green line) maize cell suspensions. Data from the early logarithmic (A and C) and late logarithmic

(B and D) phase of culture cycle are shown. The [ $^{14}\text{C}$ ]cinnamic acid consumption ( $\blacktriangledown$ ) was calculated from the radioactivity remaining in the culture medium fraction after [ $^{14}\text{C}$ ]cinnamic feeding. The incorporation of  $^{14}\text{C}$ -labelled compounds in the protoplasmic fraction ( $\bullet$ ) and Alcohol Insoluble Residue (cell wall fragments) ( $\circ$ ) is represented. The values shown are the average  $\pm$  standard deviation (n=3).

**Figure 6:** Kinetics of incorporation of  $^{14}\text{C}$ -labelled compounds (esterified and free-phenolics) into the protoplasmic polymer fraction after the addition of exogenous [ $^{14}\text{C}$ ]cinnamic acid to non-habituated (purple line) and habituated to 1.5  $\mu\text{M}$  of DCB (green line) maize cell suspensions at the early logarithmic (A) and late logarithmic (B) phase of culture cycle.  $^{14}\text{C}$ -labelled compounds were separated by TLC and assayed for radioactivity by scintillation counting. Data are expressed as percent of total polymer-esterified derivatives. Dotted line indicates the addition of  $\text{H}_2\text{O}_2$  (40 mM final concentration).

**Figure 7:** Kinetics of incorporation of  $^{14}\text{C}$ -labelled compounds esterified of alcohol-insoluble residue (cell wall fragments) after the addition of exogenous [ $^{14}\text{C}$ ]cinnamic acid to non-habituated (purple line) and habituated to 1.5  $\mu\text{M}$  of DCB (green line) maize cell suspensions at the early logarithmic (A) and late logarithmic (B) phase of culture cycle.  $^{14}\text{C}$ -labelled compounds were separated by TLC and assayed for radioactivity by scintillation counting. Data are expressed as percent of total polymer-esterified derivatives. Dotted line indicated the addition of  $\text{H}_2\text{O}_2$  (40 mM final concentration).

## SUPPORTING INFORMATION

**Figure S1:** Thin layer chromatography of  $^{14}\text{C}$ -labelled compounds (esterified and free-phenolics) of the protoplasmic fraction after the addition of exogenous [ $^{14}\text{C}$ ]cinnamic acid to non-habituated (purple line) and habituated to 1.5  $\mu\text{M}$  DCB maize cells suspensions (green line) at the early logarithmic phase of culture cycle. The position (—) of *p*-coumaric acid (Cou), ferulic acid (Fer), cinnamic acid (Cinn) and 5,5'-dehydrodiferulic acid (Di-Fer) used as external markers is shown. Compounds with low  $R_f$  are regarded as oligomers (Fry et al. 2000). + 60 min  $\text{H}_2\text{O}_2$  shows a treatment for 60 min with 40 mM  $\text{H}_2\text{O}_2$ , 120 min after [ $^{14}\text{C}$ ]cinnamic acid feeding.

**Figure S2:** Thin layer chromatography of  $^{14}\text{C}$ -labelled compounds (esterified and free-phenolics) of the protoplasmic fraction after the addition of exogenous [ $^{14}\text{C}$ ]cinnamic acid

to non-habituated (purple line) and habituated to 1.5  $\mu$ M DCB maize cell suspensions (green line) at the late logarithmic phase of culture cycle. Other details as in Figure S1.

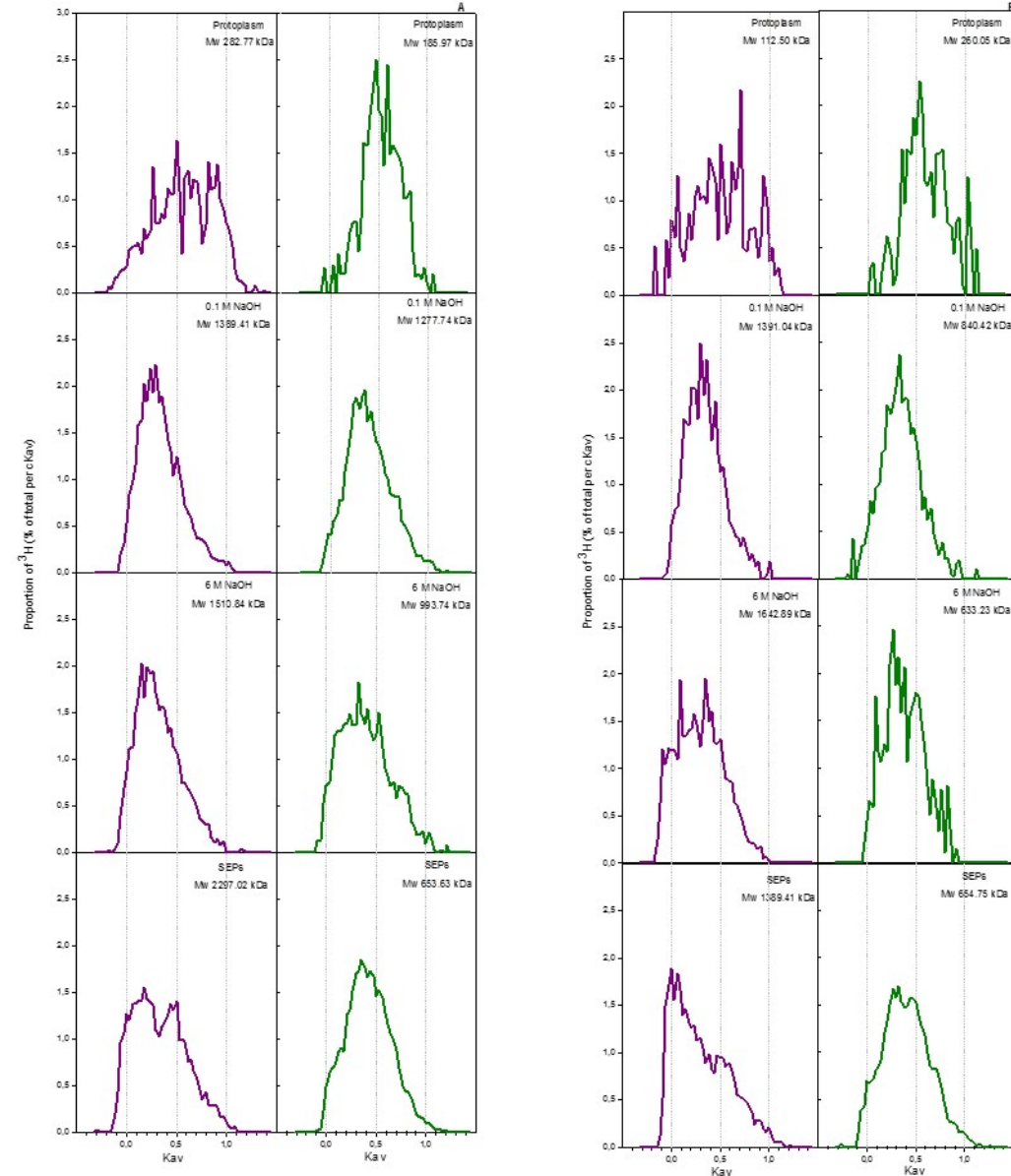
**Figure S3:** Thin layer chromatography of  $^{14}$ C-labelled compounds esterified of the alcohol-insoluble residue (cell wall fragments) after the addition of exogenous [ $^{14}$ C]cinnamic acid to non-habituated (purple line) and habituated to 1.5  $\mu$ M DCB maize cells suspensions (green line) at the early logarithmic phase of culture cycle. The position (—) of *p*-coumaric acid (Cou), ferulic acid (Fer) and 5,5'-dehydrodiferulic acid (Di-Fer) used as external markers is shown. Compounds with low R<sub>f</sub> are regarded as oligomers (Fry et al. 2000). The inset graphics show in detail the oligomers and ferulic acid dimers appearance. +60 min H<sub>2</sub>O<sub>2</sub> shows a treatment for 60 min with 40 mM H<sub>2</sub>O<sub>2</sub>, 120 min after [ $^{14}$ C]cinnamic acid feeding.

**Figure S4:** Thin layer chromatography of  $^{14}$ C-labelled compounds esterified of the alcohol-insoluble residue (cell wall fragments) after the addition of exogenous [ $^{14}$ C]cinnamic acid to non-habituated (purple line) and habituated to 1.5  $\mu$ M DCB maize cell suspensions (green line) at the late logarithmic phase of culture cycle. Other details as in Figure S3.

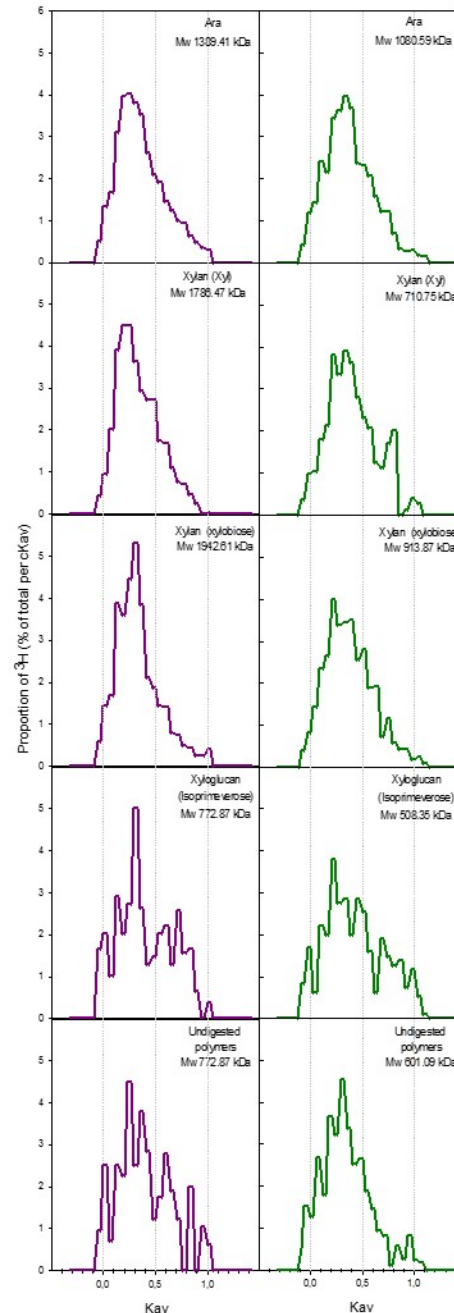
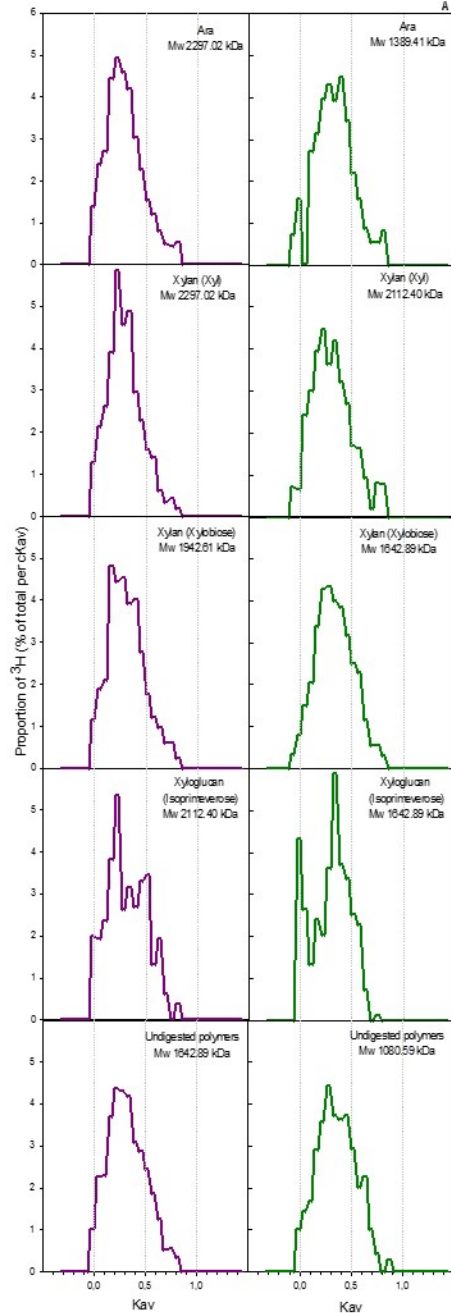
**Figure S5:** Relative expression of orthologous maize genes of several IRX genes from arabidopsis analyzed by RT-PCR of non-habituated (Snh) and habituated to 1.5 (Sh1.5) and 6  $\mu$ M DCB (Sh6) maize cell suspensions.  $\uparrow$ : more mRNA accumulation than control (Snh);  $\downarrow$  less mRNA accumulation than control (Snh). *ZmUbi*: Ubiquitin gene expression. Ubiquitine was used as the housekeeping gene due to its constitutive expression.

**Table S1.** Primers used for the analysis of maize arabinoxylan synthesis genes.

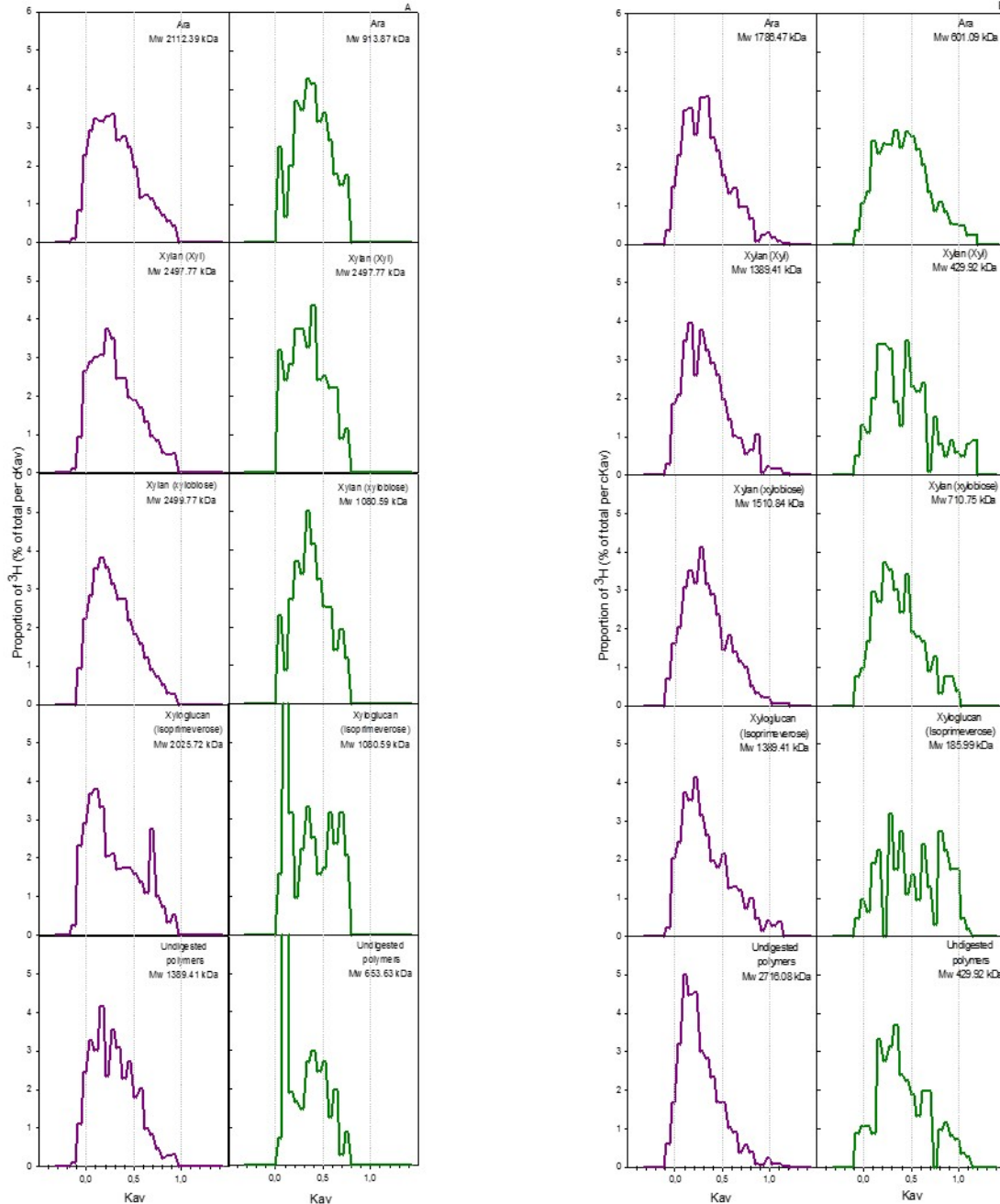




**Figure 1:** Mr distribution profile of  $^3\text{H}$ -polysaccharides: protoplasmic  $^3\text{H}$ -polymers, cell wall bound  $^3\text{H}$ -hemicelluloses extracted with 0.1 M or 6 M NaOH and soluble extracellular  $^3\text{H}$ -polymers (SEPs) obtained from non-habituated (Snh, purple line) and habituated to 1.5  $\mu\text{M}$  DCB (Sh1.5, green line) maize cell cultures. Data shown are from the early logarithmic (A) and late logarithmic (B) phase of culture cycle. Samples used were taken 300 minutes after feeding of [ $^3\text{H}$ ]arabinose, except in the case of protoplasmic  $^3\text{H}$ -polymers, collected 30 and 60 minutes after feeding of [ $^3\text{H}$ ]arabinose for Snh and Sh1.5 respectively.  $^3\text{H}$ -polymers and  $^3\text{H}$ -hemicelluloses were size-fractionated on Sepharose CL-4B. One cKav is defined as an interval of 0.01 on the  $x$ -axis. The profiles are scaled such that the area under the curve is equal (100%) in each frame. Mw values, derived from the median Kav(1/2) of the profiles and then converted to Mw via a calibration curve, are included in the text frame.

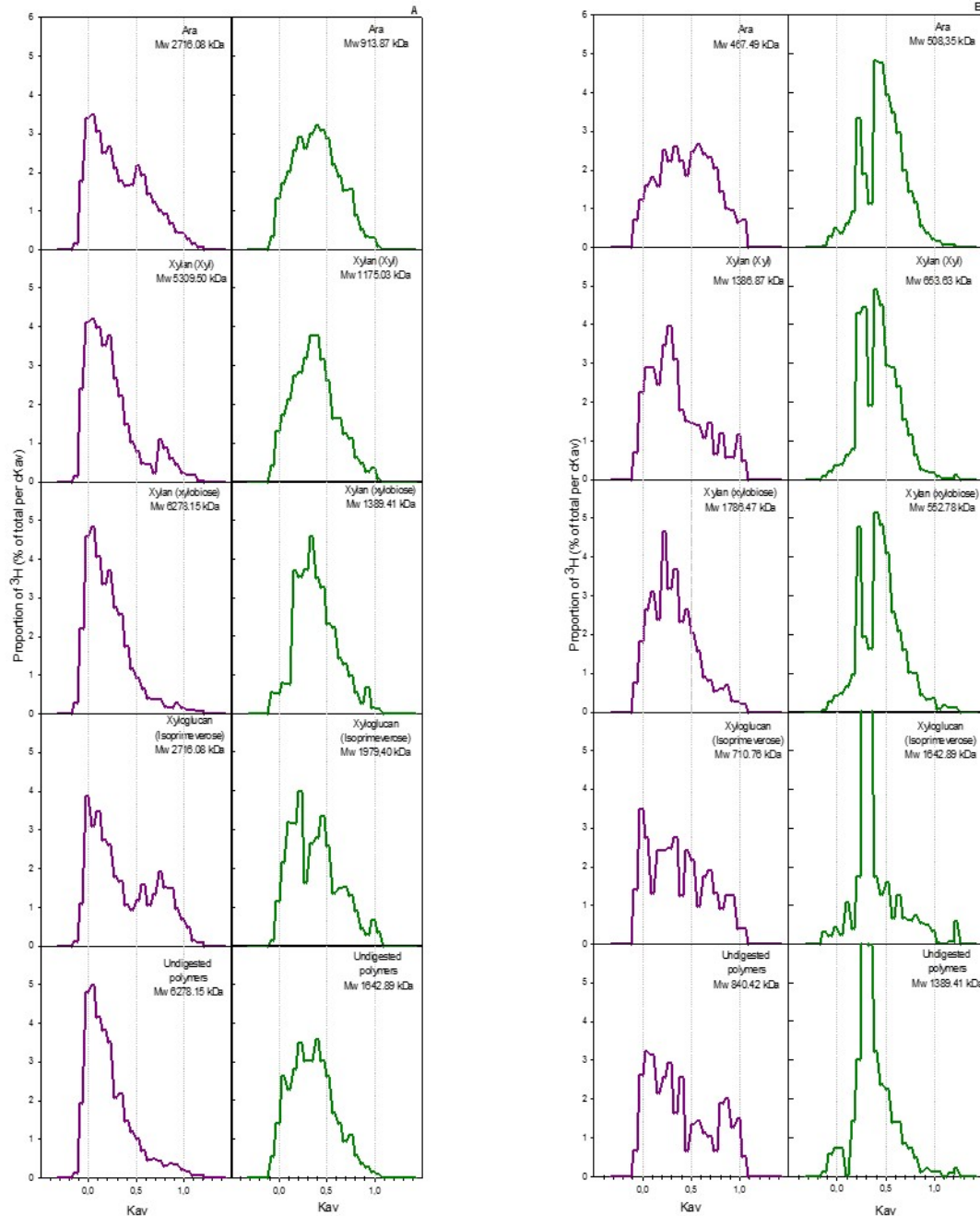


**Figure 2:** Mr distribution profile of  $^3\text{H}$ -labelled diagnostic fragments from  $^3\text{H}$ -polysaccharides: cell wall bound  $^3\text{H}$ -hemicelluloses extracted with 0.1 M NaOH obtained from non-habituated (purple line) and habituated to 1.5  $\mu\text{M}$  DCB (green line) maize cell cultures. Data shown are from the early logarithmic (A) and late logarithmic (B) phase of culture cycle. Samples used were taken 300 minutes after feeding of [ $^3\text{H}$ ]arabinose. Digest of each fraction were assayed for radioactive fragments characteristic of polymers which have arabinose residues { [ $^3\text{H}$ ]arabinose }, xylans { [ $^3\text{H}$ ]xylose } and { [ $^3\text{H}$ ]xylobiose }, xyloglucans { [ $^3\text{H}$ ]isoprimeverose } and polymers that Driselase does not digest { undigested  $^3\text{H}$ -polymers }. Other details as in Figure 1.

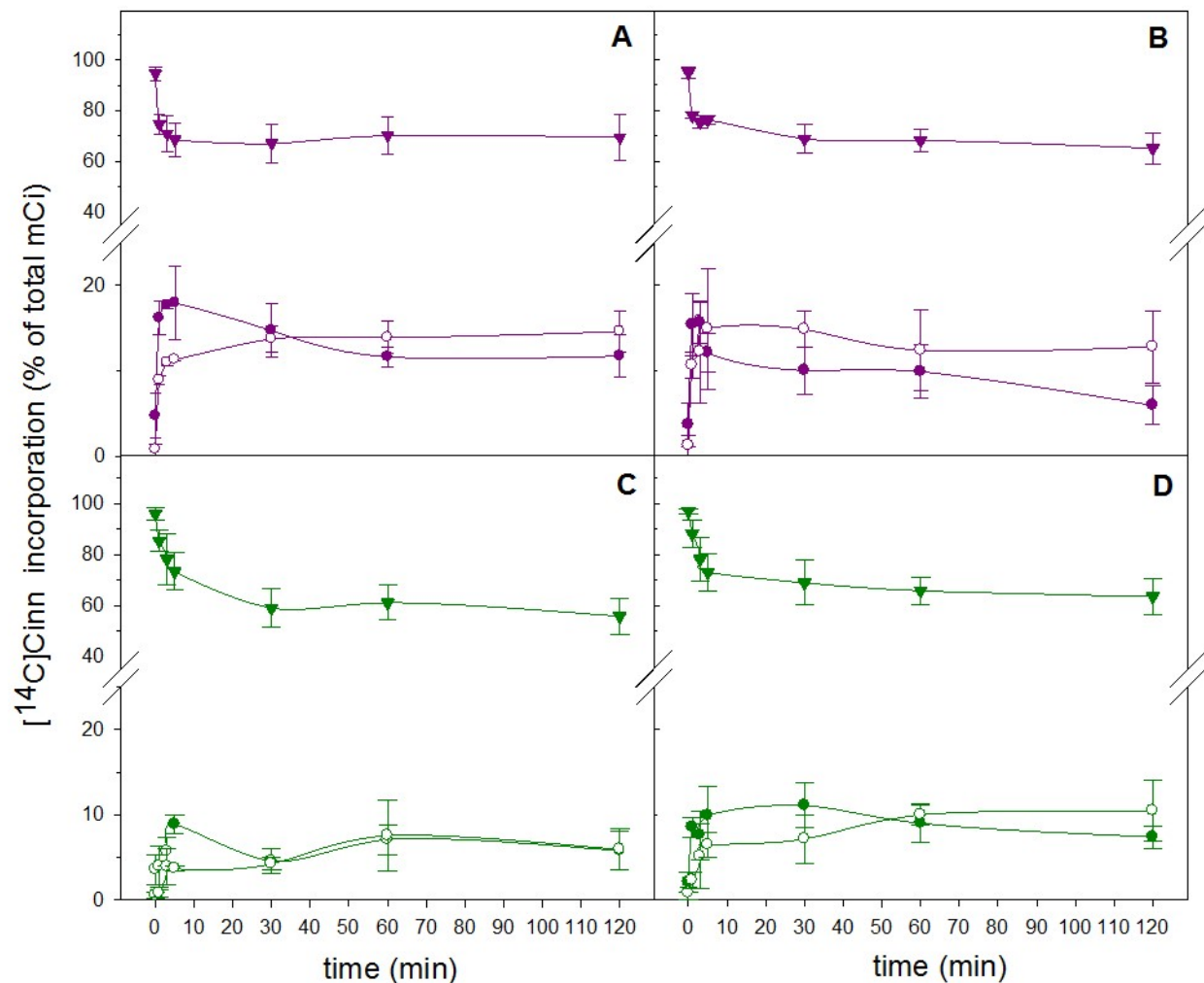


**Figure 3:** Mr distribution profile of cell wall bound  $^3\text{H}$ -hemicelluloses extracted with 6 M NaOH obtained from non-habituated (purple line) and habituated to 1.5  $\mu\text{M}$  DCB (green line) maize cell cultures. Data shown are from the early logarithmic (A) and late logarithmic (B) phase of culture cycle. Samples used were taken 300 minutes after feeding of  $^3\text{H}$ arabinose. Digest of each fraction were assayed for radioactive fragments characteristic of polymers which have arabinose residues {[ $^3\text{H}$ ]arabinose}, xylans {[ $^3\text{H}$ ]xylose} and {[ $^3\text{H}$ ]xylobiose}, xyloglucans {[ $^3\text{H}$ ]isoprimeverose} and polymers that Driselase does not digest {undigested  $^3\text{H}$ -polymers}. Other details as in Figure 1.

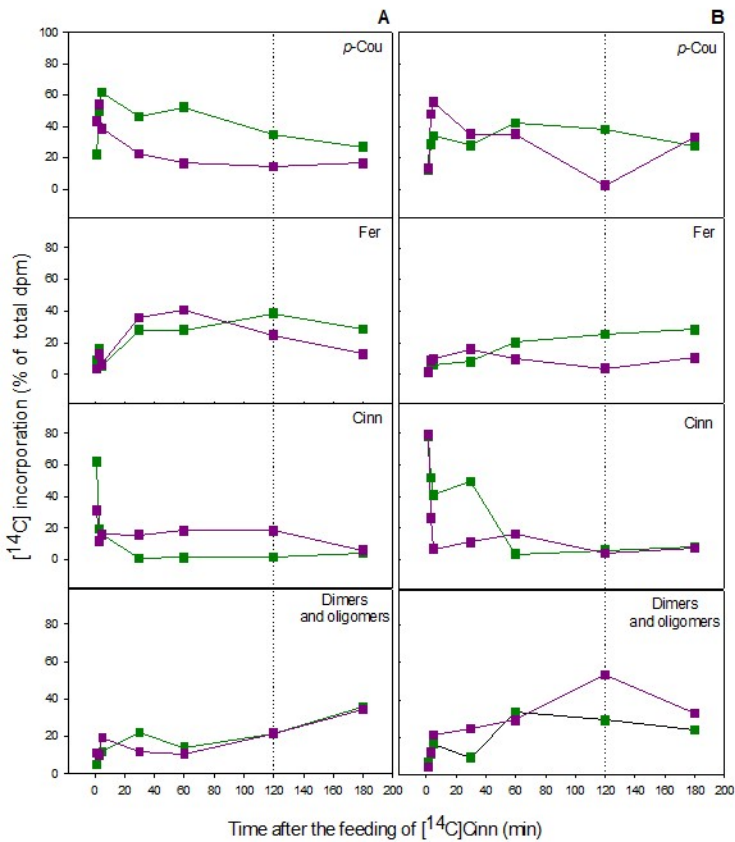




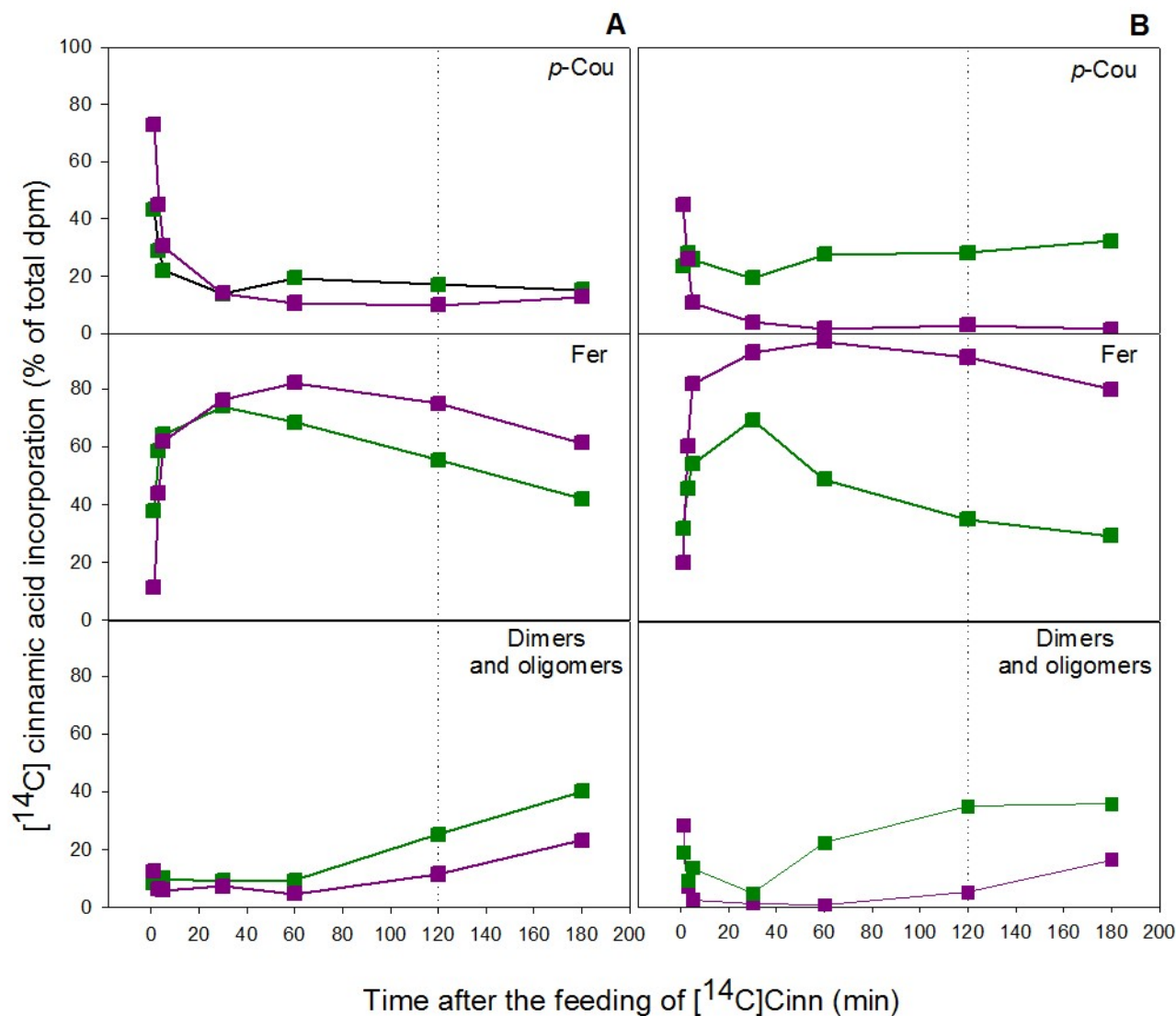
**Figure 4:** Mr distribution profile of soluble extracellular  $^3\text{H}$ -polymers obtained from the culture medium of non-habituated (purple line) and habituated to  $1.5\ \mu\text{M}$  DCB (green line) maize cell cultures. Data shown are from the early logarithmic (**A**) and late logarithmic (**B**) phase of culture cycle. Samples used were taken 300 minutes after feeding of  $^3\text{H}$ arabinose. Digest of each fraction were assayed for radioactive fragments characteristic of polymers which have arabinose residues  $\{^3\text{H}\text{arabinose}\}$ , xylans  $\{^3\text{H}\text{xylose}\}$  and  $\{^3\text{H}\text{xylobiose}\}$ , xyloglucans  $\{^3\text{H}\text{isoprimeverose}\}$  and polymers that Driselase does not digest  $\{\text{undigested } ^3\text{H-polymers}\}$ . Other details as in Figure 1.



**Figure 5:** Kinetics of consumption and incorporation of radioactivity from exogenous  $[^{14}\text{C}]$ cinnamic acid in non-habituated (purple line) and habituated to  $1.5\ \mu\text{M}$  DCB (green line) maize cell suspensions. Data from the early logarithmic (A and C) and late logarithmic (B and D) phase of culture cycle are shown. The  $[^{14}\text{C}]$ cinnamic acid consumption (▼) was calculated from the radioactivity remaining in the culture medium fraction after  $[^{14}\text{C}]$ cinnamic feeding. The incorporation of  $^{14}\text{C}$ -labelled compounds in the protoplasmic fraction (●) and alcohol-insoluble residue (cell wall fragments) (○) is represented. The values shown are the average  $\pm$  standard deviation ( $n=3$ ).

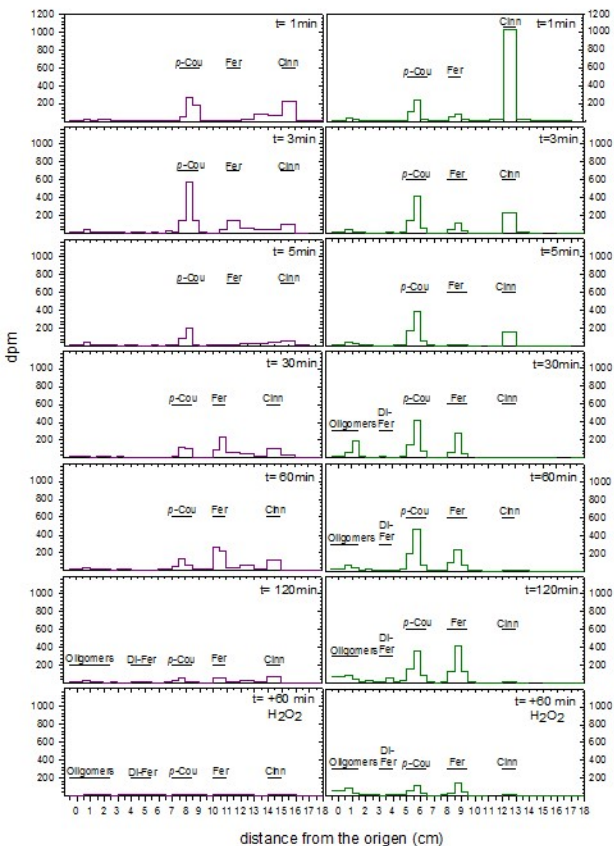


**Figure 6:** Kinetics of incorporation of  $^{14}\text{C}$ -labelled compounds (esterified and free-phenolics) into the protoplasmic polymer fraction after the addition of exogenous [ $^{14}\text{C}$ ]-cinnamic acid to non-habituated (purple line) and habituated to  $1.5\ \mu\text{M}$  of DCB (green line) maize cell suspensions at the early logarithmic (A) and late logarithmic (B) phase of culture cycle.  $^{14}\text{C}$ -labelled compounds were separated by TLC and assayed for radioactivity by scintillation counting. Data are expressed as percent of total polymer-esterified derivatives. Dotted line indicated the addition of  $\text{H}_2\text{O}_2$  (40 mM final concentration).



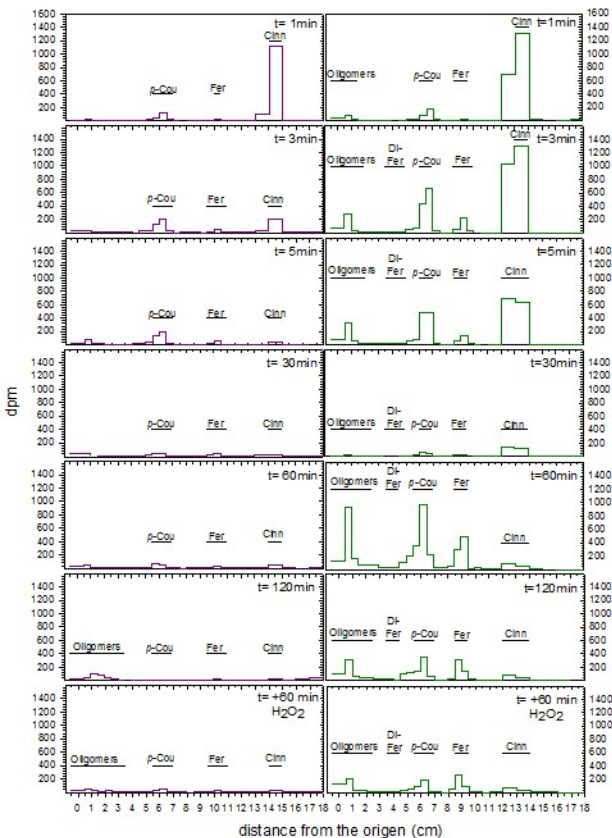
**Figure 7:** Kinetics of incorporation of <sup>14</sup>C-labelled compounds esterified to alcohol-insoluble residue (cell wall fragments) after the addition of exogenous [<sup>14</sup>C]cinnamic acid to non-habituated (purple line) and habituated to 1.5 μM of DCB (green line) maize cell suspensions at the early logarithmic (A) and late logarithmic (B) phase of culture cycle. <sup>14</sup>C-labelled compounds were separated by TLC and assayed for radioactivity by scintillation counting. Data are expressed as percent of total polymer-esterified derivatives. Dotted line indicated the addition of H<sub>2</sub>O<sub>2</sub> (40 mM final concentration).



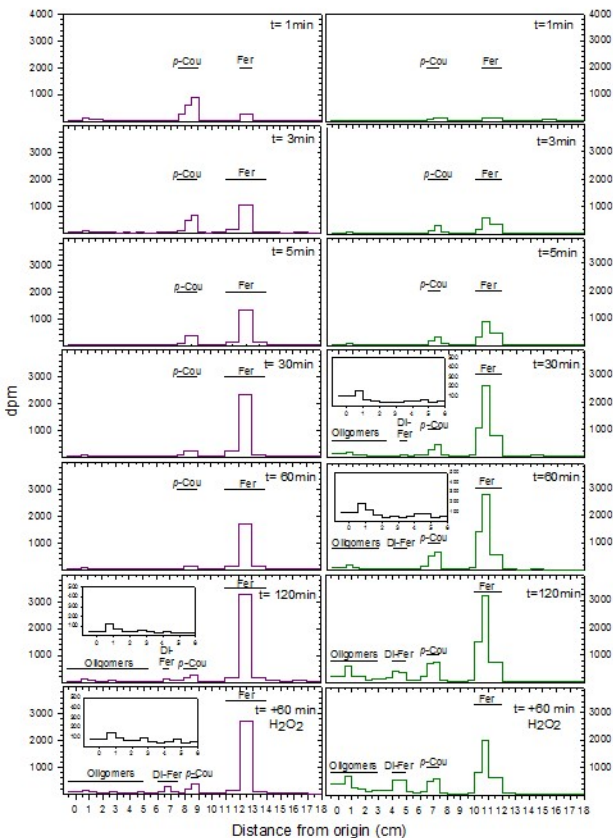


**S1:** Thin layer chromatography of  $^{14}\text{C}$ -labelled compounds (esterified and free-phenolics) on the protoplasmic fraction after the addition of exogenous [ $^{14}\text{C}$ ]cinnamic acid to non-habituated (purple line) and habituated to 1.5  $\mu\text{M}$  DCB maize cells suspensions (green line) at the early logarithmic phase of culture cycle. The position (—) of *p*-coumaric acid (Cou), ferulic acid (Fer), cinnamic acid (Cinn) and 5,5'-dehydrodiferulic acid (Di-Fer) used as external markers is shown. Compounds with low  $R_f$  are regarded as oligomers (Fry et al. 2000). + 60 min  $\text{H}_2\text{O}_2$  shows a treatment for 60 min with 40 mM  $\text{H}_2\text{O}_2$ , 120 min after [ $^{14}\text{C}$ ]cinnamic acid feeding.

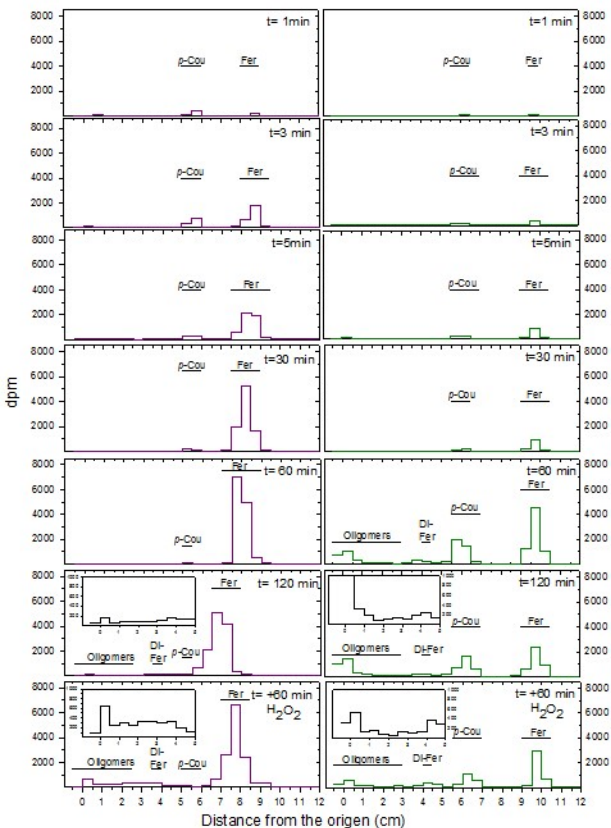




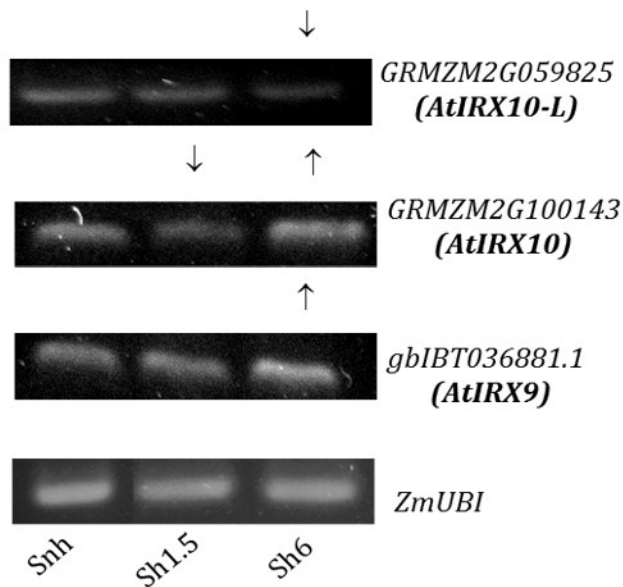
**S2:** Thin layer chromatography of  $^{14}\text{C}$ -labelled compounds (esterified and free-phenolics) on the protoplasmic fraction after the addition of exogenous  $[^{14}\text{C}]$ cinnamic acid to non-habituated (purple line) and habituated to 1.5  $\mu\text{M}$  DCB maize cell suspensions (green line) at the late logarithmic phase of culture cycle. Other details as in Figure 7.



**S3:** Thin layer chromatography of <sup>14</sup>C-labelled compounds esterified to the alcohol-insoluble residue (cell wall fragments) after the addition of exogenous [<sup>14</sup>C]cinnamic acid to non-habituated (purple line) and habituated to 1.5 μM DCB maize cells suspensions (green line) at the early logarithmic phase of culture cycle. The position (—) of *p*-coumaric acid (Cou), ferulic acid (Fer) and 5,5'-dehydrodiferulic acid (Di-Fer) used as external markers is shown. Compounds with low R<sub>f</sub> are regarded as oligomers (Fry et al. 2000). The inset graphics show in detail the oligomers and ferulic acid dimers appearance. +60 min H<sub>2</sub>O<sub>2</sub> shows a treatment for 60 min with 40 mM H<sub>2</sub>O<sub>2</sub>, 120 min after [<sup>14</sup>C]cinnamic acid feeding.



**S4:** Thin layer chromatography of  $^{14}\text{C}$ -labelled compounds esterified to the alcohol-insoluble residue (cell wall fragments) after the addition of exogenous  $[^{14}\text{C}]$ cinnamic acid to non-habituated (purple line) and habituated to 1.5  $\mu\text{M}$  DCB maize cell suspensions (green line) at the late logarithmic phase of culture cycle. Other details as in Figure 10.



**S5:** Relative expression of orthologous maize genes of several IRX genes from arabidopsis analyzed by RT-PCR of non-habituated (Snh) and habituated to 1.5 (Sh1.5) and 6  $\mu$ M DCB (Sh6) maize cell suspensions.  $\uparrow$ : more mRNA accumulation than control (Snh);  $\downarrow$  less mRNA accumulation than control (Snh). *ZmUbi*: Ubiquitin gene expression. Ubiquitine was used as the housekeeping gene due to its constitutive expression.

**Table S1.** Primers used for the analysis of maize arabinoxylan synthesis genes

Maize gene ID (arabidopsis homologous)	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
GRMZM2G100143 ( <i>IRX10</i> )	GATGCAGGCTCACCTTATCC	CGCTGCATGTCCTCGTAGTA
GRMZM2G059825 ( <i>IRX10-L</i> )	ACGTTGGTTCAGACCTTTGG	TCATTGCCGGTGTCATAGAA
gbIBT036881.1 ( <i>IRX9</i> )	TGAACTCGAGAATGCTGTGG	GCTCAATTACCCACCCTTGA